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Strategies for Recruitment and Selection of Mesenchymal Stem Cells

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Preface

The present work is a part of a wider research project which aims at the investigation of strategies for *in situ* Tissue Engineering approaches. In particular the attention has been focused on the study of signals involved in stem cells recruitment and selection.

The first part of the thesis reviews recent studies concerning the ability of Mesenchymal Stem Cells to migrate to site of damage and what signals are responsible for chemotaxis of stem cells. The second part of this thesis is articulate in three chapters describing the experimental campaign designed in order to gather information about the effects of these signals on the Mesenchymal Stem Cells. In particular my efforts have been devoted to establish the effect of some cytokines and peptides on migratory behavior of Mesenchymal Stam Cells, in terms of molecular and migratory parameters effects.

Two of three chapter, following the introduction, are relative to one scientific article about the experiment carried out to realize innovative device able to recruit and select Mesenchymal Stem Cells both in three dimensional matrix and twoo-dimensional platforms. Chapter 3 includes the article “Sustained Stromal Derived Factor-1 gradient in three-dimensional matrix as guidance for stem cells” submitted for publication to European Journal of Cell and Material and presently under revision. In chapter 5 is reported the article “Peptide activated materials to recruit circulating stem cells” to be submitted for publication.

This work carried out within the PhD program has benefited from the expert collaboration of engineers and chemists of the IIT@CRIB (Interdisciplinary Research Center on Biomaterials) as well as from the thorough and bright supervision of Professor P.A. Netti.

CHAPTER 1

INTRODUCTION

1.1 TISSUE ENGINEERING

Prospects, limits and future challenges

Regenerative medicine and tissue engineering (TE) hold the promise to solve several problems related to tissue and organ replacements. TE is based on the sapient combination of cells, material scaffolds and *in vitro* culturing conditions which aims at generating a hybrid biological/synthetic device that has to replace the functions of an injured or diseased tissue or organ.

Biomaterials play central roles in modern strategies in regenerative medicine and TE as designable biophysical and biochemical milieus that direct cellular behaviour and function (Peppas and Langer 1994; Hubbel 1995; Langer and Tirrel 2004). The guidance provided by biomaterials may facilitate restoration of structure and function of damaged or dysfunctional tissues, both in cell-based therapies and in acellular therapies. Such materials should provide a provisional three-dimensional support to interact biomolecularly with cells to control their function, guiding the spatially and temporally complex multicellular processes of tissue formation and regeneration.

Despite the early positive results, tissue engineered devices rarely have found a massive and successful clinical implementation. The commercialization, that is a major prerequisite for fitting within a wider healthcare system, of tissue engineered constructs has had alternate fortunes so far. Although tissue engineered devices could be technically implemented in clinics, it should be borne in mind that, cellular therapies are used mostly where an alternative conservative treatment cannot be pursued. Several causes may limit this. Local regulatory bodies can pose severe limitations on the use of exogenous/xenogenous cells and off-the-shelf availability is not

straightforward. In fact, the isolation of cells from patients, their amplification and subsequent *in vitro* culture are the most critical steps of the whole process, being highly costly and time consuming. Nevertheless, a large body of research is constantly focused on finding alternative and safer strategies which might eventually lead to a successful clinical implementation of TE.

The TE paradigm is to isolate specific cells through a small biopsy from a patient, to grow them on a three-dimensional biomimetic scaffold under precisely controlled culture conditions, to deliver the construct to the desired site in the patient's body, and to direct new tissue formation into the scaffold that can be degraded over time (Lee and Mooney 2001). TE also offers unique opportunities to investigate aspects of the structure-function relationship associated with new tissue formation in the laboratory and to predict the clinical outcome of the specific medical treatment. In order to achieve successful regeneration of damaged organs or tissues based on the TE concept, several critical elements should be considered including biomaterial scaffolds that serve as a mechanical support for cell growth, progenitor cells that can be differentiated into specific cell types, and inductive growth factors that can modulate cellular activities (Putman and Mooney 1996; Heath 2000).

The biomaterial plays an important role in most TE strategies (Hubbel 1995). For example, biomaterials can serve as a substrate on which cell populations can attach and migrate, be implanted with a combination of specific cell types as a cell delivery vehicle, and be utilized as a drug carrier to activate

specific cellular function in the localized region (Marler et al. 1998; Murphy and Mooney 1999).

Thus a mayor goal is to develop new culture-based approaches, using advanced biomaterials, that more closely mimic what the body already does so well and promote differentiation of pluripotent cells or propagation of specialized adult stem cells without loss of "stemness" (Lutolf et al. 2009). An increasing emphasis on design principles drawn from basic mechanism of cell- matrix interactions and cell signalling has now set the stage for the successful application of biomaterials to stem-cell biology. This strategy has the potential to revolutionize our understanding of extrinsic regulators of cell fate, as matrices can be made using technologies that are sufficiently versatile to allow recapitulation of features of stem-cell microenvironments, or niches, down almost to the molecular detail (Lutolf and Hubbel 2005).

In the body, adult stem cells reside within instructive, tissue-specific niches that physically localize them and maintain their stem-cell fate (Scadden 2006; Morrison and Spradling 2008). To shed light on the mechanisms that regulate stem cells, approaches that allow the study of stem-cell function in response to isolated components of a complex system — that is, models that simplify it — are crucial. Biomaterials approaches, in combination with other technologies such as microfabrication and microfluidics, are well suited to assist studies of stem-cell biology through the creation of evolving systems that allow key variables to be systematically altered and their influence on stem-cell fate analysed. Thus, biomaterials technologies provide the exciting possibility of deconstructing and then reconstructing niches, allowing

quantitative analysis of stem-cell behaviour in a manner not previously possible.

Biomaterials can be designed to act as carriers for the local delivery of stem cells, support cells or molecular niche cues. The biomaterials may markedly improve the impact of transplanted stem-cell populations. Many of the concepts described for *in vitro* use above could find useful application *in vivo*. For example, materials could be designed as multifunctional stem-cell microenvironments that affect tissue regeneration on multiple levels, including the following: delivering stem cells in a protective material and enhancing viability; delivering support cells to increase the numbers and stimulate the function of endogenous stem cells; delivering diffusible cytokines to promote the mobilization of endogenous cells involved in repair, such as those that form blood vessels; displaying regulatory proteins to enhance survival and to stimulate self-renewal and expansion of the transplanted cells; and displaying regulatory proteins to stimulate tissue-specific differentiation for the purpose of large-scale tissue regeneration. Probably the spatial and temporal control of these features would enhance their utility in tissue regeneration, improving tissue function and overcoming the adverse effects of disease or ageing (Lutolf et al. 2003; Wang et al. 1999). The inhibitory or stimulatory molecules or drugs that might increase stem-cell numbers or function when delivered to the niche. This could be achieved by forming a scaffold that leads to timed drug (small chemical) or biomolecule delivery near a stem-cell niche or by targeted delivery of soluble microparticles or nanoparticles as carriers of such bioactive niche components (Zhang et al. 2008). Biofunctional polymer particles can now be

engineered to be efficient in such applications. Specifically, they can be functionalized so that they bind to specific molecules on cells, are responsive to environmental signals such as proteases secreted by cells, or are delivered encapsulated in a manner that leads to temporally controlled release or cellular uptake (Rothenfluh et al. 2008; Gu et al. 2008). The most challenging, but perhaps the ultimate, biomaterials goal is to create multi-component, injectable materials designed to act as *de novo* niches *in vivo*. Artificial niches would need to incorporate appropriate 'homing' signals that could attract endogenous stem cells and localize them by means of known cell–cell or cell–matrix adhesive interactions. Then, once localized to these artificial niches, the cells would need to be exposed to tethered signals that control stem-cell function, in particular expansion by self-renewal division. Neighbouring vascular cells and neural cells would need access. Upon injury, the up-regulation and release of proteases would enable the newly formed stem cells to escape the niche and contribute to differentiation and tissue regeneration.

The aim of this thesis is also to understand how the cells perceive the information provided from the scaffold, and how the information should be presented.

The ideal scaffold should be "the mirror" that replicates as Nature provides the cells with multiple signals and orchestrates their spatial arrangement and temporal evolution. In fact, stem cell fate is not simply governed by the presence or absence of these signals, but also on the way they are presented to the surroundings. Instructing a cell to perform a specific task requires to establish a communication between the synthetic device and the cell itself.

Therefore the knowledge of the cellular language (the set of signals, as well as the way they are presented) and the logic (i.e. cascade of intracellular signalling pathways, leading to changes in gene expression and affecting most aspects of cell behaviour) that govern the response to exogenous stimuli become of crucial importance. Therefore novel processing and manipulation technologies will be developed and optimized in order to encode bioactive cues with predetermined spatial placement and timing. The selection of signals has to be motivated on 1) the signalling pathway we intend to stimulate and 2) the possibility of effective encoding of the signals, i.e. preserving their biostability. To this aim, short peptidic sequences, protein domains or even synthetic drugs, which are known to exert the desired effects on cellular activity, should be preferentially selected as biochemical cues.

1.1.1 *In situ* Tissue Engineering

In situ tissue engineering could be a promising approach useful to bypass several limitations represented by some basic steps involved in the traditional concept of TE. In situ Tissue Engineering offers the possibility to recruit adult stem cells and progenitor cells that reside at the site of scaffold transplantation and then providing them the adequate stimuli in order to induce differentiation and tissue regeneration, therefore bypassing the delicate procedures of cell isolation and *in vitro* culture.

This strategy could solve ethical and regulatory issues related to cell source. As matter of fact, in the traditional TE strategies, cells may be isolated from autologous, allogenic, or xenogenic sources. Thus, autologous cells would

seem to be the best choice, but cell isolation from patients in need of treatment can cause additional normal tissue morbidity. In order to obtain sufficient numbers for transplantation, in vitro proliferation is essential, which may cause undesirable phenotype change (Zhang and Lodish 2005). The pluripotency of stem cells may decrease during in vitro culture. In addition, the cost of in vitro expansion of stem cells is very high, since a battery of growth factors is needed for the propagation procedures. The economic and multi-week expansion period present important challenges to these clinical procedures. Finally, while adult stem cells attract much attention because of their pluripotency, this characteristic decreases during in vitro culture using conventional two-dimensional (2D) culture conditions(Banfi et al. 2000; Muraglia and Quarto2000). An alternative cell source could be endogenous stem cells. Indeed, a regenerative medicine approach for tissue repair focused on the direct manipulation of endogenous adult stem cells is very appealing. There are several advantages to the use of endogenous stem cells for tissue repair. First, using endogenous stem cells avoids the immunocompatibility issues that attend the use of allogenic and xenogenic cells. Second, it is easier, safer, and more efficient to use endogenous stem cells for tissue repair to expand and re-implant autologous cells. Third, only a single surgical intervention is required, rather than two surgeries several weeks apart. Finally, the process of recruiting endogenous stem cells offers both regulatory and economic advantages relative to ex vivo approaches. The utilization of endogenous stem cells may be enhanced in two ways. One strategy is to mobilize the endogenous stem cells into the circulation. For example, it is reported that granulocyte colony-stimulating

factor (G-CSF) mobilizes stem and progenitor cells from the bone marrow into the peripheral blood, from which they can 'home' into the lesion site in the brain and have a protective or restorative effect (Borlongan and Hess 2006).Also, the mobilized endogenous stem cells are showing promising outcomes for cardiac repair (Orlic et al. 2001). A second strategy is to enhance the recruitment of endogenous stem cells into the lesion site for tissue regeneration. Several factors, such as growth factors and cytokines have shown chemotaxic effects on stem cells.

Concerning in situ tissue engineering, recent works focused on the incorporation/delivery of Stromal derived factor-1 (SDF-1) within/from scaffolds in order to recruit stem cells in vivo. In particular, Schantz et al. (2007) sequentially delivered Vascular Endothelial Growth Factor (VEGF), SDF-1 and Bone Morphogenetic Protein-6 (BMP-6) in a Polycaprolacton scaffold implanted in a rat model, demonstrating stem cell infiltration with evidences of angiogenesis and tissue precursor formation. Bladergroen et al. (2009) created an heparinized collagen scaffold loaded with SDF-1and than implanted in a mouse model. They demonstrated that the release of SDF-1 was effective in recruiting hematopoietic stem cell. Thevenot et al. (2010) developed a system to constantly deliver SDF-1 within a PLGA scaffold implanted in a mouse model. Following this approach, they reported a significant recruitment of stem cells, with evidences on increased healing and angiogenesis and concomitant reductions in inflammation and scaffold encapsulation. These works demonstrated the effectiveness of the use of SDF-1 within a TE scheme. However, the influence of SDF-1 on the dynamics of stem cell migration is not fully achieved.

Other researcher reported the possibility to *in vivo* recruit MSC by a PCL scaffold loaded with transforming growth factor β1 (TGFB) for potential application of in situ chondrogenesis (Huang et al 2002). Zhao and colleagues (2008) created an injectable, in situ crosslinkable semisynthetic ECM-like hydrogel for (Hepatocyte growth factor) HGF delivery and MSC recruitment. However also in these studies there are not information about how MSC perceive these signals and how these signals influence cell migratory behaviour.

1.2 STEM CELLS

Stem cells, responsible for maintenance of homeostasis and repair of tissues, are increasingly being considered as an important source in cell—based therapeutic strategies for regeneration of various tissue owing to their characteristic of self-renewal and multipotency. Because of their unique capacity to regenerate functional tissues, stem cells are an attractive "raw material" for multiple application in TE field. Recent findings have shown that stem cells exist in most tissues and that stem cells tissue specificity may be more flexible than originally thought. Although the potential for producing novel cell-based products from stem cells is large, currently there are no effective technologically relevant methodologies for culturing stem cells outside the body, or for reproducibly stimulating them to differentiate into functional cells. Understanding what parameters are important in the control of stem cells self-renewal, migration and lineage commitment is thus necessary to guide the development of bioprocesses for the ex vivo culture of stem cells and their derivates.

While embryonic stem cells generate diverse tissues, adult stem cells are specialized and essential for tissue maintenance and repair throughout life (Lanza et al 2006). In adulthood, tissue homeostasis and regeneration are critically dependent on both the self-renewal and the differentiation capacity of stem cells. However, to fully exploit this clinical potential, we must increase our knowledge of the regulatory mechanisms that govern stem cell behavior. To date only a few adult stem cell types are approved for clinical use. Bone marrow transplants that harbor hematopoietic stem cells (HSCs) have saved the lives of numerous leukemia and lymphoma patients and skin transplants have significantly alleviated disfigurement and increased the function of burn victims. To overcome the hurdles inherent in enlisting adult stem cells therapeutically, stem cell biologists are addressing fundamental questions regarding the precise cell-intrinsic and cell-extrinsic regulation of key stem cell. It is of paramount importance to understand how a multitude of diverse biochemical and biophysical cues present in spatial vicinity of cells should affect cellular behaviour.

The key function of stem cell niches is to maintain a constant number of slowly dividing stem cells during homeostasis by balancing the proportions of quiescent and activated cells. On insult (that is, injury, disease or damage), stem cells exit the niche and proliferate extensively, self-renew and differentiate to regenerate the tissue. Within the niche, stem cells are thought to be exposed to complex, spatially and temporally controlled biochemical mixtures of soluble chemokines, cytokines and growth factors, as well as insoluble transmembrane receptor ligands and extracellular matrix (ECM) molecules In addition to understanding this biochemical signalling regulatory

network, it is key to appreciate the biophysical properties of the niche, including matrix mechanical properties and architecture (topographical cues), to elucidate the role of niche elements (Discher et al. 2009; Guilak et al. 2009).

As described in several excellent reviews (Fuchs et al. 2004, Scadden 2006, Moore and Lemischka 2006, Li and Xie 2005) mammalian niches have been identified and characterized in multiple tissues including the skin (in the bulge region of the hair follicle), intestine (in the epithelium), brain (in the hippocampus), bone marrow (on the endosteal surface and near blood vessels), and muscle (beneath the muscle fibre basal lamina). Stem cells are in intimate physical contact with support cells which provide short-range signals via soluble factors as well as via membrane-bound proteins. Stem cells are also surrounded by an extracellular matrix (ECM), a protein- and sugar-rich crosslinked gel network that provides structure and organization as well as biochemical and mechanical signals (Fig. 1). Blood vessels are often found near niches (or are believed to constitute niches themselves, e.g., in the central nervous system (Palmer et al. 2000) or bone marrow (Heissing et al. 2002 Kiel and Morrison 2006), presumably serving to transport long-range signals and as a conduit for recruitment of circulating cells into the niche.

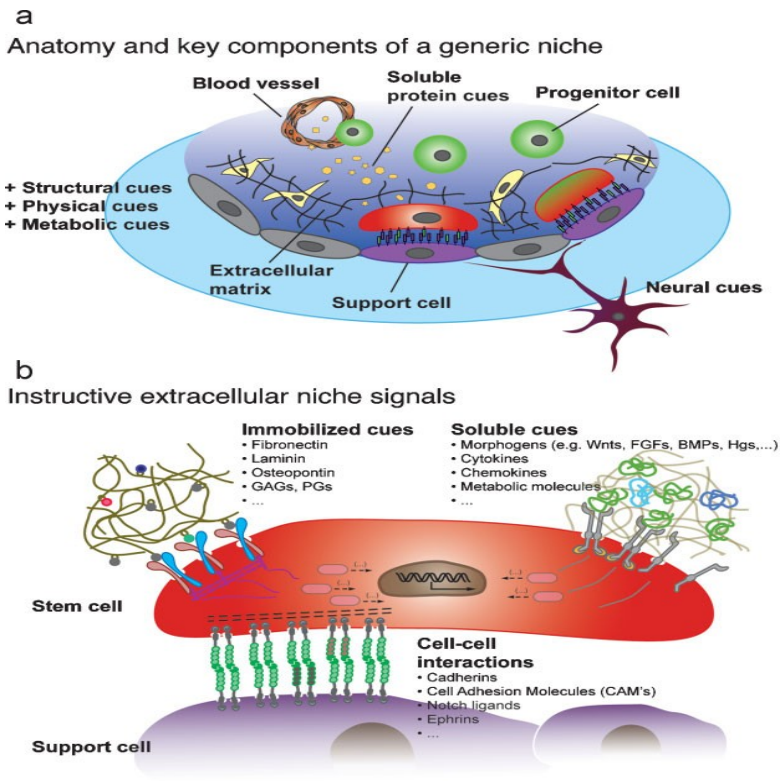
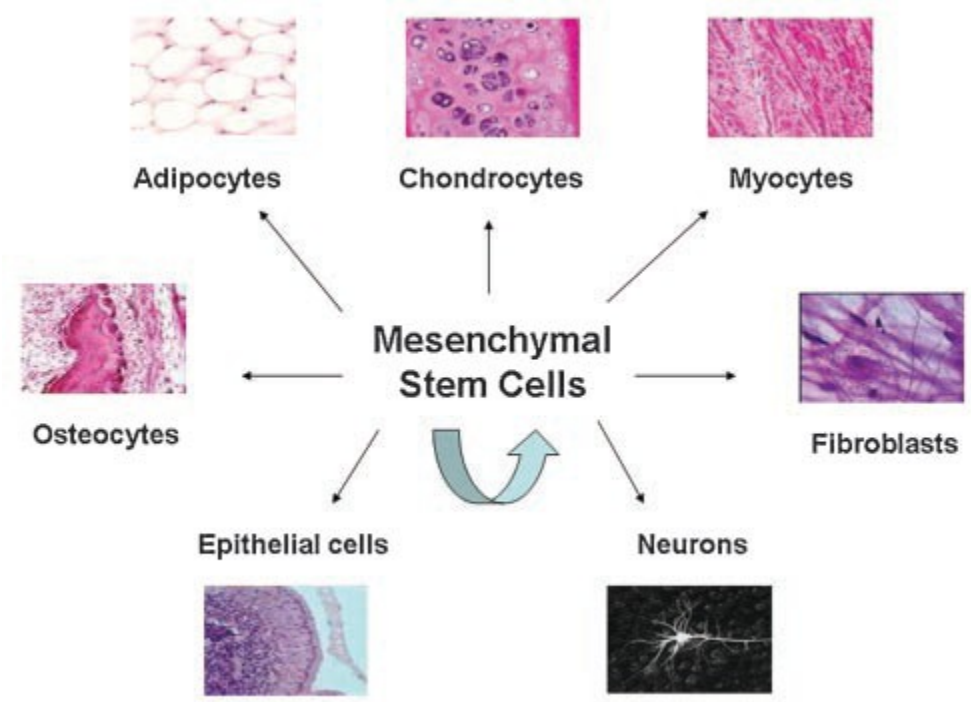


Figure 1. Architecture and composition of a stem cell niche. Adult stem cells are located within instructive microenvironments comprised of complex mixtures of extracellular cues delivered by support cells in close proximity (a). The main components of a niche are support cells and their secreted transmembrane cell–cell adhesion proteins, soluble factors, and the surrounding ECM (b).

1.3.1 Characteristics and therapeutic potential of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a subset of nonhematopoietic stem cells, characterized by their ability of self-renewal and differentiation into multiple cell types, including osteoblasts, adipocytes and chondrocytes. MSCs were first described by Friedenstein in 1970s as fibroblast-like, plastic-adherent cells that can be expanded in vitro (Friedenstein et al. 1974). MSCs are easy to isolate, with low immunogenicity, multidifferentiation potentials, and lack of ethical controversy.



Pluripotent capacity of MSC to differentiate into mesodermal and non-mesodermal cell lineages, including osteocytes, adipocytes, chondrocytes, myocytes, fibroblasts, epithelial cells, and neurons

Several works have shown that mesenchymal progenitors are also present, although at low frequencies, in adult peripheral blood (Zvaifler et al. 2002) and in term cord blood (Erices et al. 2000); besides a population of MSCs has been isolated from fetal blood, liver, and bone marrow in the first-trimester of pregnancy (Campagnoli et al. 2001). Again Kuznestov and colleagues (2001) demonstrate the presence of stem cells of extravascular mesodermal tissues in the circulating peripheral blood, capable to generate at least three phenotypes of the so called stromal system. However the origin of these cells, the manner by which they gain the access to the blood stream and they physiological role remain to be properly addressed. Recent studies assume that tissue-specific stem cells circulate under steady-state conditions in the

peripheral blood and maintain a pool of stem cells, and their number increases during stress/tissue injury (Kucia et al.2004).

It has been demonstrated that MSCs in the circulation could migrate to the site of tissue damage, such as bone or cartilage fracture (Murphy et al. 2003), myocardial infarction (Barbash et 2003, Shake et al. 2002), and ischemic cerebral injury (Wang et al 2002). Therapeutic values of MSCs have been demonstrated in animal models of acute lung injury (Ortiz et al. 2007), liver injury (Liang et al 2009), myocardial infarction (Zohlnhofer et al. 2008), diabetes (Urban et al. 2008), stroke (Shyu et al. 2004), limb ischemia (Iwase et al.2005), acute renal injury (Patschan et al. 2006) and sepsis (Nemeth et al 2009). In order to participate in repair and regeneration, MSCs have to be mobilized and then migrate to the target sites and integrate with the local tissues. Many studies have been focusing on the mechanisms for MSCs to migrate to injured tissues. Those research have identified some important molecular mechanisms, including chemoattractants, paracrine factors, membrane receptors, and intracellular signaling molecules.

Recent studies revealed some key molecules and signalling pathways, which could be potential targets for modulation of MSC migration in wound healing, damage repair and regeneration.

Studies showed contrasting results and discrepant conclusion. Different types of MSCs and heterogeneity of MSCs used may account for the discrepancy (Karp et al. 2009). Different signalling mechanisms may underlie migration of different types of stem cells (Li et al. 2009). In addition, confluency and passage number of cultured MSCs have been shown to influence migration of MSCs (Karp et al. 2009; De Becker et al 2007;

Rombouts et al. 2003). Heterogeneity between different sources or different passages of MSCs could have significant impact on MSC migration. Migration of MSCs is controlled by complicated signal networks. Understanding the molecular mechanisms of MSC migration will benefit optimization of stem cell therapies. Regulation of the signalling pathways and extracellular matrix will help to develop strategies to facilitate targeting of transplanted MSCs as well as endogenous MSCs to injured tissues (Mannello et al. 2006; Zhu et al. 2006).

Chemotactic factors and membrane receptors

Several growth factors and their receptors may be involved in MSC migration. Hepatocyte growth factor (HGF) was up-regulated at sites of liver damage (Kollet et al. 2003; Jankowski et al. 2003). Following myocardial ischemia and reperfusion in a rat model, HGF and its high-affinity receptor c-met were unregulated (Ono et al 1997). In an animal model of myocardium infarction, increased HGF expression was found in the injured heart (Kucia et al. 2004). Human bone marrow- and circulating blood-derived MSCs expressed functional c-met receptors, and could be strongly attracted by HGF gradients. This chemotactic response could be significantly inhibited using the specific c-met blocking agent K-252a, suggesting that HGF-c-met signaling regulates migration of MSCs (Son et al. 2006). Up-regulation of HGF expression in multiple injured tissues may induce MSCs to migrate to the HGF-rich environment of lesions. Other growth factors, including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), epidermal growth factor (EGF) and angiopoietin- 1(Ang-1), were also reported

to be chemotactic to MSCs (Ponte et al. 2007; Forte et al. 2006; Fiedler et al 2002; Fiedler et al 2006; Tamama et al. 2006). MSCs express receptors for those growth factors at a moderate to high level, including platelet-derived growth factor receptor (PDGF-R), insulin-like growth factor 1 receptor (IGF1-R), epidermal growth factor receptor (EGF-R) and Ang-1 receptor (Ponte et al. 2007). Thus, multiple growth factor-receptor axes may mediate MSC migration.

Tumor necrosis factor-α (TNF-α) is an important inflammatory cytokine presented at most injury sites with inflammation. TNF-α induces and directs migration of rat bone marrow MSCs in vitro (Fu et al. 2009). Extracellular high mobility group box 1 (HMGB1) is a cytokine that plays a role in the processes of inflammation, tissue injury and regeneration. Meng et al (Meng et al. 2008) showed that HMGB1 could act as a chemoattractant for MSCs in a dose-dependent manner. Monocyte chemotactic protein-1 (MCP-1), a chemokine involved in recruitment and activation of macrophages during inflammation, stimulates MSC migration to ischemia in the rat brain (Wang et al. 2002), as well as to tumor (Dweyer et al. 2007).

SDF-1–CXCR4 Axis:

Stromal-derived factor 1(SDF-1), or CXC ligand 12, is a member of a large family of related chemotactic cytokines, called “chemokines”, which was first identified as a lymphocyte and monocyte specific chemo-attractant under both normal and inflammatory conditions (Moser et al. 2004). Subsequently it has been demonstrated that MSCs express CXCR4, the receptor for SDF-1, and therefore SDF-1/CXCR4 axis has been implicated in the migration of MSC in a series of studies (Li et al. 2007; Bhakta et al. 2006; Wynn et al.

2004). Those studies suggest that SDF-1/CXCR4 axis was required for migration of human bone marrow MSCs and cord blood MSCs. CXCR4 antagonist AMD3100 significantly inhibited chemotaxis of MSCs toward SDF-1(Wynn et al. 2004; Son et al. 2006). Rat bone marrow MSCs were shown to migrate towards SDF- 1 gradient in a dose-dependent manner (Li et al. 2007; Ji et al. 2004). In a rat model, SDF-1-CXCR4 was shown to mediate homing of transplanted MSCs to injured sites in the brain (Ji et al. 2004).

CX3CL1-CX3CR1 Axis:

CX3C ligand 1 (CX3CL1, also called fractalkine) and CX3C receptor 1(CX3CR1) were reported to mediate MSC migration response. Using an in vitro micromultiwell chemotaxis chamber assay, human bone marrow MSCs were found to migrate towards fractalkine gradients, and such response was abrogated using anti- CX3CL1 mAb (Sordi et al. 2005). In a rat model of left hypoglossal nerve injury, interaction of fractalkine-CX3CR1 plays an important role in directed migration of transplanted rat MSCs to impaired sites in the brain (Ji et al. 2004). It should be noted that other chemokines and chemokine receptors, including CXCL16-CXCR6, CCL3-CCR1 and CCL19- CCR7, were also reported to participate in MSC migration (Sordi et al. 2005).

Intracellular signaling pathways

Extracellular signals through membrane receptors induce varieties of intracellular signaling pathways, which result in changes in cell motility and migration direction.

PI-3K/AKT signaling pathway:

Phosphoinositide 3-kinase (PI-3k)/ Akt signaling pathway is involved in SDF-1-mediated cell migration of hematopoietic progenitor cells and primary marrow CD34+ cells (Wang et al. 2009). Genetically modified BMSC that over-express Snail showed more migration advantages, and disruption of the PI-3k-dependent pathway using specific PI-3k inhibitor, wortmannin, reduced Snailmediated BMSC migration (Zha et al. 2007). SDF-1α or bFGF-induced MSCs migration was attenuated by PI3k/Akt inhibitor LY294002 or wortmannin (Li et al. 2007; Schmidt et al. 2006).

MAPK/ERK1/2 signaling pathway:

Mitogen-activated protein/ extracellular signal-regulated kinase 1/2 (MAPK/ERK1/2) signal pathway is involved in the expression of a wide variety of genes controlling migration. ERK1/2 may mediate SDF-1-induced cell mobilization (Liao et al. 2005; Zhang et al. 2006). Several studies have shown that MAPK/ERK1/2 was involved in MSC migration. Yun et al. (2009) demonstrated that stable thromboxane A2 (TxA2) mimetic U46619 strongly stimulated migration of human adipose tissue-derived MSCs (hADSCs) through activation of ERK and p38 MAPK. U46619- induced MSC migration was abrogated if the cells were pretreated with the MAPK/ERK kinase (MEK) inhibitor U0126 or the p38 MAPK inhibitor SB202190.

Wnt3a signaling pathway:

Wnt signaling is involved in the metastasis of many kinds of cancer cells (Pinto et al. 2005; Qiang et al. 2005). Wnt3a promoted the migration capacity of rat MSCs in transwell migration and wound healing assays through β-

catenin nuclear translocation (Shang et al. 2007). Wnt3a antibodies significantly reduced migration of MSCs.

Jak/STAT signaling:

PDGF-induced migration of hADSCs was completely blocked by a pretreatment with c-Jun N-terminal kinase (JNK) inhibitor SP600125, but not with MEK inhibitor U0126 and p38 MAPK inhibitor SB202190 (Kang et al. 2005). Janus kinase (Jak)/ signal transducer and activator of transcription (STAT) signaling was originally shown to be downstream signaling of interferons during the inflammatory response (Aaronson et al. 2002; Platanias et al. 2005). Jak/STAT pathway activation is required for cell migration in Drosophila (Silver et al. 2005). MSC migration in response to SDF-1 stimulation resulted in Jak2/STAT3 pathway activation, and inhibition of the pathway using WP1006, a Jak2/ STAT3 pathway inhibitor, significantly inhibited MSC migration (Gao et al. 2009). Activation of Jak2/STAT3 pathway led to focal adhesion kinase (FAK) and paxillin activation which resulted in reorganization of actin filament and cytoskeleton, thus promoting MSC migration.

1.4 CELL MIGRATION AND CHEMOTAXIS

Cell migration orchestrates morphogenesis throughout embryonic development (Gilbert et al. 2003). During gastrulation, for example, large groups of cells migrate collectively as sheets to form the resulting three-layer embryo. Subsequently, cells migrate from various epithelial layers to target locations, where they then differentiate to form the specialized cells that make up different tissues and organs. Analogous migrations occur in the

adult. In the renewal of skin and intestine, fresh epithelial cells migrate up from the basal layer and the crypts, respectively. Migration is also a prominent component of tissue repair and immune surveillance, in which leukocytes from the circulation migrate into the surrounding tissue to destroy invading microorganisms and infected cells and to clear debris.

Migration contributes to several important pathological processes, including vascular disease, osteoporosis, chronic inflammatory diseases such as rheumatoid arthritis. In general cell migration can be usefully conceptualized as a cyclic process (Lauffemburger and Horwitz 2006). The initial response of a cell to a migration-promoting agent is to polarize and extend protrusions in the direction of migration. These protrusions can be large, broad lamellipodia or spike-like filopodia, are usually driven by actin polymerization, and are stabilized by adhering to the extracellular matrix (ECM) or adjacent cells via transmembrane receptors linked to the actin cytoskeleton. These adhesions serve as traction sites for migration as the cell moves forward over them, and they are disassembled at the cell rear, allowing it to detach. Interestingly, the movement of cell sheets shows some features of single-cell migration; however, the polarization extends across the sheet. Although many aspects of this picture are shared among different cell types, the details can differ greatly. For example, these steps are observed most distinctly in slow-moving cells such as fibroblasts, but are not as obvious in fast-moving cells such as neutrophils, which seem to glide over the substratum. In addition, a cell's migratory behavior depends on its environment. Somatic cells migrating in vivo, for example, show large single protrusions and highly directed migration, in contrast to the multiple small protrusions they display on

planar substrates; and cancer cells can modify their morphology and nature of migration in response to environmental changes (Knight 200; Friedl 2003).

Orchestrated movement of cells in a particular direction to a specific location is a central process for tissue development, maintenance, and repair. Cell movement is controlled by extracellular matrix substrates and by secreted molecules such as growth factors and cytokines. The understanding of cell migration and chemotactic mechanisms may lead to the novel therapeutic strategies for tissue repair or regeneration.

During adult life, cell movement plays an important role in tissue repair, wound healing and the functioning of the immune system. In these processes, the spatiotemporal patterns of cell movement are under the control of extracellular guidance cues produced by surrounding cells and tissues. These cues may be present in gradients of diffusible and/or matrix-bound signaling molecules and direct cell movement toward or away from their sources.

In vivo tissue regeneration depends on migration of stem cells into injured areas and their differentiation into specific cell types that are necessary to set up new tissue. In the same way, also in TE strategies application, in order to induce a functional and stable integration between host tissue and scaffold, is necessary the migration of stem/precursor cells.

Several techniques have been developed to characterize the movement of both individual cells and whole populations. The wound assay, (Majack et al. 1994) the Teflon fence assay (Pratt et al. 1984) and the phagokinetic track assay (Lewis et al. 1987) all measure cell movement on uniform substrates using information from only the beginning and end of the experiment. Cells

migrate from known positions defined by the system geometry and are fixed for viewing at the end of the experiment to determine the net distance traveled during the experiment. Videomicroscopy techniques have provided the ability to continuously monitor cell behavior during migration and introduced the possibility of tracking discrete cell motion (Dow 1987). To date, little work has been done to characterize cell motion in response to gradients of surface-bound molecules. Boyden et al. (1962) studied cell response to gradient stimuli; however, the lack of specific information about the gradient generated in the porous mesh and the inability to observe cell motion during experiments have limited the utility of the Boyden chamber.(Boyden et al. 1962) Movement of anchorage-dependent cell types can be attributed to random motility, chemotaxis, haptotaxis, or the sum of their effects. Random motility is the persistent random movement of a cell that over a short time period, called the persistence time, tends to persist in one direction, but over a long period of time will result in no directional displacement. Random motility can be altered by both soluble factors and adhesion molecules on the surface (Boyden 1962) but remains inherently nondirectional. Chemotaxis is a biased and persistent movement of cells in response to a gradient of soluble stimuli in conjunction with random motility, which can be either attractive or repulsive. Haptotaxis is essentially the same phenomenon as chemotaxis, except in response to a surface-bound gradient. The modeling of cell movement in response to chemotactic and haptotactic gradients is critical for differentiating random and directed components of cell motion.

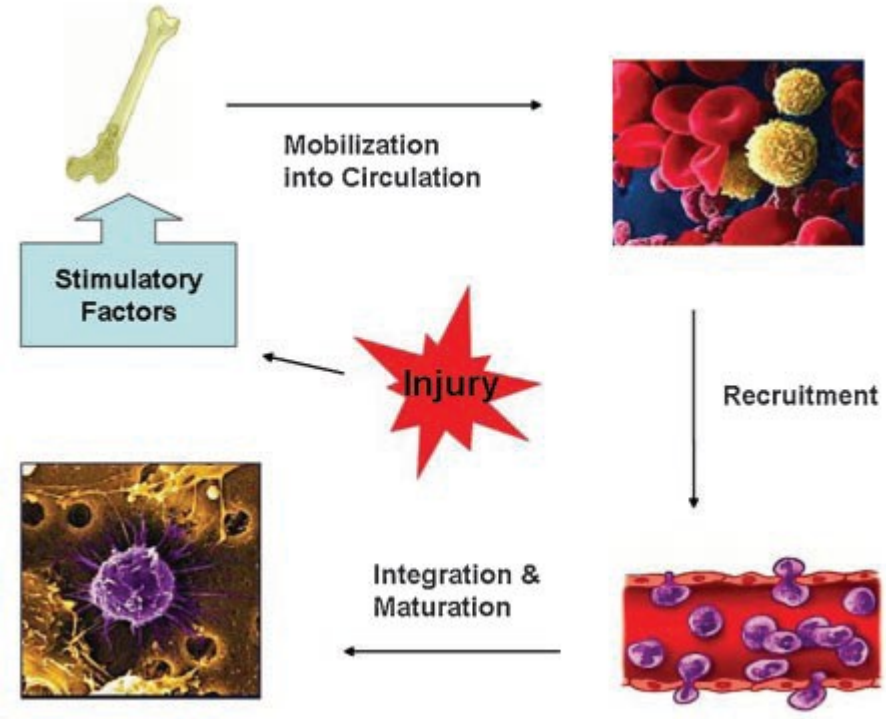
1.4.1 Homing ability of MSC

The ability of injured adult tissue to regenerate implies the existence of cells capable of proliferating, differentiating and/or functionally contributing to the reparative process.

MSCs reside in specialized niches within various tissues, and it has been shown that bone marrow, bone and spleen are all sites of engraftment (18-21) It has also recently been reported that a very small number of MSCs consistently circulate in peripheral blood under stationary conditions, and that this circulating pool is greatly increased under injury conditions.

MSC appear to be reservoir of reparative cells that lack tissue-specific characteristics and can potentially be mobilized and differentiate into cells of a connective tissue lineage under different signals, such as damage from trauma, fracture, inflammation, necrosis and tumors (Pountos et al. 2006). Recent studies (Mansilla et al. 2006; Wang et al. 2006) suggest that injury/trauma might initiate the mobilization of MSC into peripheral blood. These circulating stem cells are believed to home to the damaged or pathological tissues in a mechanism similar to leukocyte recruitment to sites of inflammation that involves adhesion molecules such as selectins, chemokine receptors and integrins. The migration of MSC from the circulation into injured or unhealthy tissues and the resulting therapeutic response have been documented (Granero-Moltò et al. 2009; Ortiz et al 2003; Chen et al. 2001; Wu et al. 2003). Increasingly, studies tend to conclude that the beneficial effects of MSC can be due to two possible mechanisms of reparative action (Fox et al. 2007): not only the in situ differentiation of MSC to become normal constituents of the host cytoarchitecture and supporting

stroma after recruitment to the injury site (Lee et al. 2009), but also to act via a paracrine mechanism. The latter is an emerging concept whereby MSC are believed to possess the capacity to home to the site of injury, and subsequently secrete a broad spectrum of paracrine factors that are both immunoregulatory and function to structure the regenerative microenvironment (Caplan et al 2007).



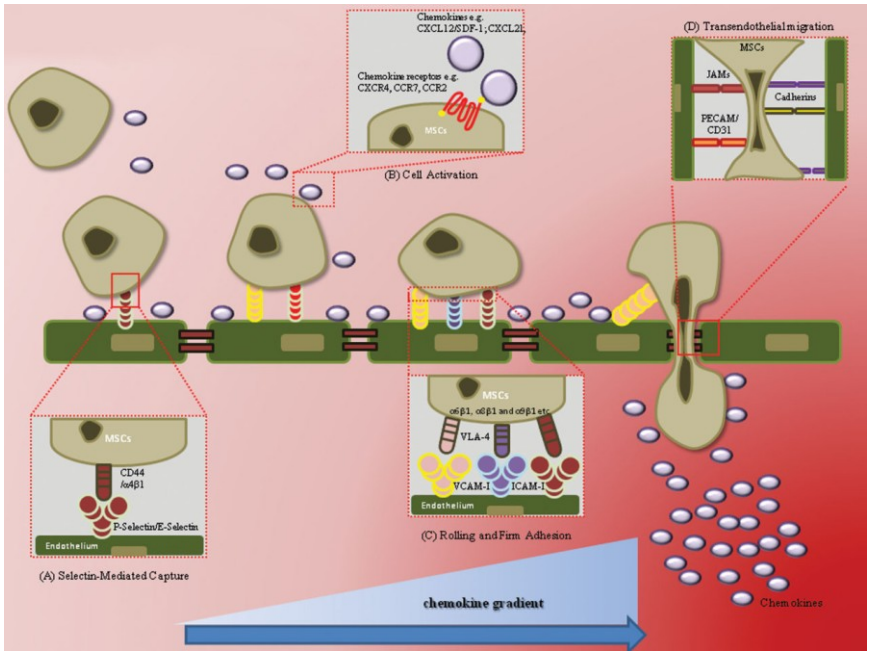
Injury in the periphery releases stimulatory factors that cause mobilization of MSCs from bone marrow into the circulation. At the site of injury, certain molecules expressed on the endothelium causes recruitment of MSCs, where they transmigrate from blood vessels and undergo in situ maturation and integrate into the injured tissue to bring about healing.

Homing is essentially the process by which cells migrate to and engraft in the tissue in which they will exert functional and protective effects. This homing feature of MSCs means that the presumed complications associated with intramuscular or site-specific injection of stem cells, such as ossifications, is avoided, and systemic intravenous delivery with the potential for multiple dosages is possible.

However, only a small number of studies addressed factors and mechanisms that mediate mesenchymal stem cells migration and information on the activity and relative potency of various potential chemotactic factors is limited. Directing MSC migration toward injury sites is a potential strategy for tissue regeneration, understanding characteristics of MSC migratory behaviour is necessary for TE. Although some chemotactic factors for MSC have been identified, additional candidates may be more effective. A number of important biological phenomena involve the interplay between cellular traction and directed migration. Chief among them are mesenchymal morphogenetic processes such as tumor stroma formation (Dvorak et al.), embryogenesis (Stopak et al.), teratoma formation (Grabel et al.), and wound contraction (Greiling et al.) in which directed migration both affects and is affected by tractional restructuring of the extracellular matrix (ECM) through a complex feedback mechanism (Oster et al.). Cell migration may be directed by spatial gradients of soluble factors (i.e., chemotaxis) and/or fibril alignment within the ECM (i.e., contact guidance).

During inflammation, the recruitment of inflammatory cells requires a coordinated sequence of multistep adhesive and signaling events, including selectin-mediated rolling, cell activation by chemokines and cytokines, activation of integrins, integrin-mediated firm adhesion on endothelium, transendothelial migration, and finally the migration/invasion in the extracellular matrix involving integrin-dependent interactions and matrix-degrading proteases (Imhof et al. 2004, Luster et al. 2005). It is well known that migratory direction follows a chemokine density gradient. The increase in inflammatory chemokine concentration at the site of inflammation is a key

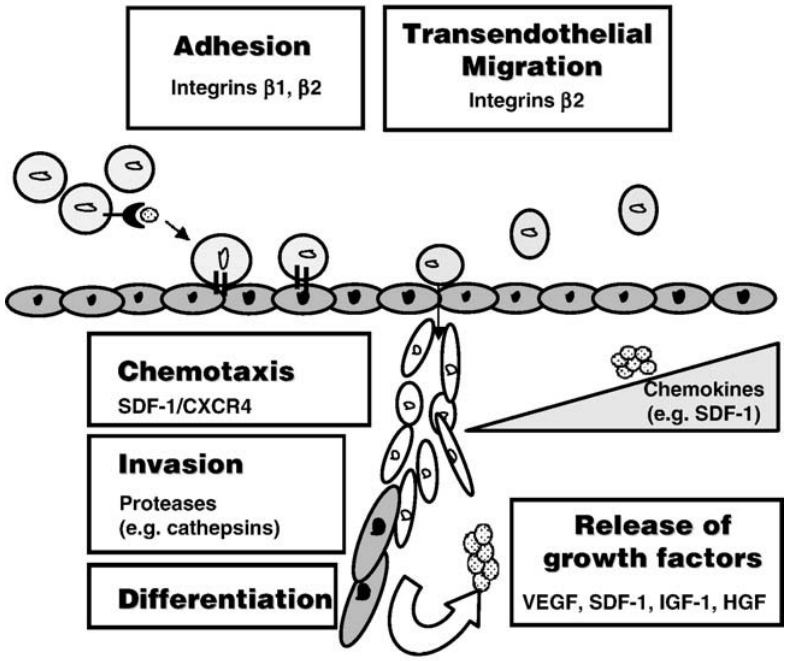
mediator of trafficking of MSC to the site of injury. Chemokines are released after tissue damage and MSC express several receptors for chemokines (Spring et al. 2005). Activation by such chemokines is also an important step during trafficking of MSCs to the site of injury.



Proposed mechanisms involved in the homing and trafficking of mesenchymal stromal cells to sites of tissue injury after infusion. Abbreviations: ICAM, intercellular adhesion molecule; JAMs, junctional adhesion molecules; MSCs, mesenchymal stromal cells; PECAM, platelet-endothelial cell-adhesion molecule; PGE, prostaglandin E2; VCAM, vascular cell-adhesion molecule; VLA, very late antigen. (Lusein et al. STEM CELLS 2010;28:585–596

The homing efficiency of MSCs has been reported to be greatly influenced by the variation in protocols currently used to isolate and culture expand populations to significant numbers required for in vivo use. It has been suggested that subculturing of MSCs may potentially lead to changes in their phenotype that effects MSC homing (Kemp et al. 2005) and progressive subculturing has been associated with a decrease in expression of adhesion molecules, the loss of chemokine receptors, including CXCR4, and a subsequent lack of chemotactic response (Son et al. 2006, Honczarenko et al. 2006). The initial homing events involve the processes of rolling and

tethering upon the endothelium between E- and P-selectin (considered as critical molecules for the rolling process) (Kansas et al. 1996). These are constitutively expressed by bone marrow endothelial cells and on endothelium in inflamed tissue (Schweitzer et al. 1996). Physiologic selectin receptors constitutively express sialylated residues such as PSGL-1 and CD44 (Lowe et al. 2002). CD44 is known to be highly expressed by MSCs. Rolling is subsequently followed by arrest and firm adhesion, with chemokines receptors expressed on the surface of endothelium ligating to respective chemokines and activating integrins, such as very late antigen- 4 (VLA-4) (also known as a4b1-integrin) (Peled et al. 2000), which in turn bind to their ligands mediating firm adhesion. Ruster et al. (2006) also reported that MSCs bind to endothelial cells in a P-selectin dependent manner and that rolling MSCs engage VLA-4 and vascular cell-adhesion molecule one (VCAM-1) to mediate firm adhesion to the endothelium. Firm adhesion is followed by transendothelial migration between endothelial cells via the action of junctional adhesion molecules (JAMs), cadherins, and platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), mediating translocation to the extracellular matrix where they adhere to the extracellular matrix through molecules such as collagen, fibronectin via a1 integrins, hyaluronic acid, and CD44.



Multistep process of progenitor cell homing and engraftment. Recruitment and incorporation of progenitor cells into ischemic or injured tissue requires a coordinated multistep process including adhesion to the endothelium, transendothelial migration, chemotaxis, matrix degradation and invasion and in situ differentiation. The factors which are proposed to regulate the distinct steps are indicated.

Chemokine-mediated MSC activation and their role in MSC biology

The role that chemokines and their receptors play in the targeting of leucocytes to areas of inflammation, infection or injury is well characterised (Miyasaka & Tanaka, 2004). As chemokine receptors are expressed on the cell surface of MSC, and their stimulation has been shown to induce cell migration, it seems likely that they play a similar role in directing MSC. MSC have been shown to express a variety of chemokine receptors, and to date CCR1, CCR4, CCR6, CCR7, CCR9, CCR10, CXCR4, CXCR5,CXCR6, CX3CR1 have been detected on human MSC (Wynn et al, 2004; von Luttichau et al, 2005; Sordi et al, 2005; Honczarenko et al, 2006; Ruster et al, 2006) with CCR2, CCR5, CXCR4, CX3CR1 being present on their rat counterparts (Ji et

al, 2004). It is not clear why the reported chemokine receptor repertoire of MSC has been inconsistent as the isolation and culture conditions are largely similar. It may be that the heterogenic nature of a typical MSC population obscures the detection of a distinct receptor repertoire. Alternatively, because the level of expression can be relatively low, the antibodies used may not have been sensitive enough to detect receptor expression. Nevertheless, the functionality of the various chemokine receptors has been demonstrated using conventional in vitro assays of chemokine-mediated MSC migration and chemokine-mediated increases in the intracellular concentration of calcium using an appropriate ligand (Kortesidis et al, 2005). As such, it will be important to define the molecular events governing chemokine receptor expression. Chemokine-mediated MSC migration has also been demonstrated in vivo. After MI, levels of CXCL12 protein have been observed to rise significantly in the left ventricle of mice. Its expression was restricted to cardiomyocytes and blood vessels in the infarct zone but not in remote areas of the myocardium (Abbott et al, 2004). Abbott et al (2004) administered BM-derived MSC intravenously into mice 48 h after inducing a MI by suture of the left anterior descending coronary artery or, as a control, in sham operated animals. They observed MSC migration within 72 h to the left ventricle only in animals that developed infarcted tissue. The importance of CXCL12 and its receptor, CXCR4, in this migration was confirmed via the administration of a specific CXCR4 receptor antagonist, AMD3100, which significantly inhibited MSC migration to the infarct site. Furthermore, when the myocardium was transduced with an adenoviral vector containing CXCL12, which led to a 2Æ5-fold increase in CXCL12 expression, MSC

detection in the heart was significantly increased. These data suggest that CXCL12 interacting with CXCR4 was critical in the migration of MSC to the infracted heart but was not sufficient to induce homing in the absence of injury (Abbott et al, 2004). In support of these findings, CXCL12 levels in humans have also been observed to rise in patients after MI. In these patients, circulating levels of MSC fluctuated and it is interesting to speculate that the fluctuations were caused by CXCL12-induced recruitment of MSC to the damaged myocardium as part of the body's response to injury (Wang et al, 2006). An important role for chemokine involvement in mediating MSC migration to the brain is also evident. After ischaemic brain injury, the level of CCL2 was observed to increase significantly in ischaemic brain tissue extract (Wang et al, 2002a). The brain tissue extract was chemotactic for MSC in vitro and this migration was significantly diminished in the presence of a neutralising CCL2 antibody and is thus likely to be mediated by its receptor CCR2, which was found to be expressed on MSC in this study (Wang et al, 2002a). CCL3 and CXCL8 may also be important agents that mediate MSC migration to damaged cerebral tissue (Wang et al, 2002b). In addition to their role in mediating cell migration, chemokines may also play important autocrine and paracrine roles. CXCL12 promotes the growth, survival and development of MSC (Kortesidis et al, 2005). MSC are known to be able to synthesise this chemokine, which may thus act in an autocrine manner via CXCR4 (Kortesidis et al, 2005). Similarly, the anti-proliferative effects of MSC on T lymphocytes may be via chemokines, such as CCL1, acting in a paracrine manner either on the T lymphocytes or via the recruitment of regulatory T-lymphocytes that subsequently induce T-cell

anergy (Batten et al, 2006). Chemokines are also recognized as primary inducers of integrin upregulation following their interaction with their cell surface receptors and various downstream signalling events. Integrins are known to mediate the firm adhesion of leucocytes to endothelial cells and play an important role in their transendothelial migration. It is likely they play a similar role for MSC.

1.5 GROWTH FACTORS AND CYTOKINES

Tissue development is regulated through the interplay of a variety of signals, including soluble signaling molecules, insoluble ligands, mechanical cues and cell-cell interactions. Numerous peptides and proteins involved in this signaling possess a biological activity that marks them as potential therapeutics. Soluble growth factors and immobilized ligands can regulate the adhesion, migration, proliferation and differentiation of various cell types. Materials play a fundamental role in all tissues engineering approaches. Materials create and maintain a space for tissue formation, provide mechanical support to the forming tissue, deliver inductive molecules or cells to the site of interest and provide cues controlling the structure and function of the newly created tissue (Putnam and Mooney 1996). To effect this latter function, materials are frequently designed to cue cells via the presentation of peptides or proteins that bind to cell surface receptors and trigger a desired cell response. One approach to direct the process of tissue formation is the incorporation of bioactive components, including growth factors and peptides mimicking the function of extracellular matrix (ECM) molecules, into biomaterials (Hubbel 1999). Advances in material engineering have led to

new modes of presenting these molecules to control the cell response and new tissue development. A large number of amino acid sequences have been identified in ECM molecules that specifically bind to cell receptors and mediate the adhesion, proliferation, differentiation and migration of the cells. In addition, a number of proteins have been identified that are secreted by cells into the surrounding fluids, and subsequently bind to the same or other cell populations, via cell surface receptors, to affect similar ranges of cellular processes. The identification of these various peptides and proteins has created the possibility of utilizing synthetic peptides, proteins purified from a natural source, and recombinantly manufactured proteins to regulate tissue regeneration. The peptide/protein may either be presented in an immobilized form from the surface of a material to interacting cells or be released from a material to interact with the cell in a soluble form.

1.5.1 Growth factor delivery

The development of appropriate delivery vehicles for growth factors will be crucial for their clinical utility. Growth factors, owing to their control of many biological processes, are finding wide-spread use in the regeneration of many tissue types. Typically, recombinant versions of the desired proteins are manufactured and delivered in solution form, either systemically or via direct injection into the tissue site of interest. However, growth factors typically have a short half-life once they are introduced into the body and are rapidly eliminated (Edelman et al.1993). This is problematic, as the target cell population must often be exposed to factors throughout the entire course of repair, or at least for an extended period. To address this challenge,

controlled delivery systems that incorporate the growth factors into polymeric biomaterials have been developed to prolong the tissue exposure time and to maintain growth factor stability. In addition to the duration of tissue exposure, the amount and timing can be crucial to the biological response. The release rate of growth factors from polymers is typically controlled by the diffusion of the factor or polymer degradation Langer 1990). Among the most commonly used materials are synthetic polymers such as poly(L-lactide) (PLA), poly(glycolide) (PGA) and their copolymers poly(lactide-co-glycolide) (PLGA), in the physical forms of microspheres or sponges (Richardson et al. 2001). Hydrogel-forming materials, including collagen (Ishikawa et al 2003), alginate (Lee et al. 2001), polyethylene glycol (PEG) (Burdick et al. 2002) and PLAPEG (Saito et al. 2003), are also frequently used. To address the instability of proteins immobilized within polymers, a variety of stabilization techniques have been developed (Zhu et al. 2001, Sohier et al. 2003). Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis, as it is a potent mitogen for endothelial cells (ECs) and induces EC migration and sprouting by upregulation of several endothelial integrin receptors (Ferrara et al. 2003).

Various polymeric materials have been used for the controlled release of VEGF, including PGA, alginate and fibrin (Wong et al. 2003). It has recently been noted that the VEGF release rate from polymers can be responsive to the local mechanical environment (lee et al. 2001), and this may be crucial in applications involving a mechanically dynamic environment (e.g. heart tissue). Fibroblast growth factor 2 (FGF-2), another well-studied angiogenic factor, elicits diverse biological effects on numerous cell types, including ECs,

and has been used in clinical trials to induce angiogenesis (Khurana and Simons 2003). FGF-2 has been incorporated into various polymers, such as gelatin (Iwakura et al. 2003), collagen (Pieper et al. 2002), chitosan (Ishihara et al. 2003) and PLA (Lee et al. 2002). FGF-2 reversibly binds to heparin-like molecules and heparin sulfate proteoglycans. The dominant growth factor delivery approach in angiogenesis utilizes single growth factors, but this may not be the ideal approach to replicate this complex process. Angiogenesis results from a complicated series of interactions involving different cytokines, growth factors, cells and proteases, acting in a consecutive, concerted or synergistic manner (Ennett and Mooney 2002). Recent advances in polymeric delivery systems allow one to locally and controllably deliver multiple growth factors with controlled doses

and rates of delivery. Delivery of VEGF and platelet-derived growth factor (PDGF) at distinct rates and doses results in a dramatic increase in the maturity of engineered vessel networks (Richardson 2001). Similarly, delivery of FGF– PDGF combinations synergistically induces stable vascular networks, whereas single growth factors are unable to maintain these newly formed vessels (Cao et al. 2003).

The transforming growth factor (TGF) family of proteins have an essential role in bone formation through the regulation of osteoprogenitor and osteoblast proliferation and differentiation (Lu et al. 2001). A variety of materials have been used for TGF-b1 delivery, including PLGA-PEG and coral (Dermes et al. 2002), and combined TGF-b1 and insulin-like growth factor (IGF) release from PLA carriers accelerates osteotomy healing (Kandziora et al. 2002) However, the optimal conditions for TGF-b1 release during bone regeneration

have yet to be defined. Bone morphogenetic proteins (BMP), members of the TGF superfamily, appear to act as differentiation factors, causing mesenchymal cells to differentiate into bone-forming cells. Recombinant human BMP-2 and BMP-7 are currently in clinical use as osteoinductive agents (Wozney 2002). Several carriers for BMP have been developed, including collagen (Akamaru etal 2003), PLGA (Bessho et al 2002) PLA–p-dioxanone– PEG block copolymers (Saito et al 2002), PLA-PEG (Saito et al. 2003) and PEG hydrogels. These systems allow control over the BMP release rate and promote pre-osteoblast differentiation and mineralization in vitro and ectopic bone formation in vivo [11]. The therapeutic potential of several growth factors in wound healing has long been recognized. PDGF is the first mediator to appear at the wound site and is active in all stages of the healing process. Delivery of PDGF by a carboxymethylcellulose-based gel (Regranex1) is employed for the treatment of diabetic foot ulcers and was the first growth factor system for TE to be approved by the Food and Drug Administration (FDA) (Nagai et al. 2002)

1.5.2 Signalling immobilization

Insoluble ECM molecules clearly regulate local cellular activity, and many functions of the ECM can be mimicked by small peptide fragments of the entire molecules (Lutolf et al.2003 A, Lutolf et al.2003 B) These fragments can be produced synthetically and covalently coupled to synthetic polymers so as to present them in a solid-state form, upon which their activity often depends, and to infer biological activity to the synthetic materials. Cellular attachment to ECM molecules is crucial for the survival, growth and

determination of a differentiated phenotype for anchorage-dependent cells. Many of these processes are mediated through ECM–integrin receptor binding. The integrin binding capability of ECM molecules has been mapped to specific oligopeptide sequences within ECM proteins, and these sequences include RGD, IKVAV and YIGSR (Pierschbacher et al. 1984, Graf et al. 1987). Advantages of employing short peptides containing these signaling motifs to modulate cell function, rather than utilizing whole adhesive proteins (e.g. fibronectin), include the ease and reproducibility of synthesizing peptides, as compared with isolating ECM molecules from a natural source. Utilization of only a small fragment of an ECM molecule may also allow one to target an interaction with a specific cell population (Hubbel et al. 1999). A limitation to this approach, however, is that the biological activity of short peptide sequences is often substantially lower than that of the complete protein, owing at least partially to the absence of complementary domains that are involved in integrin binding (Yang et al. 2001). Utilizing larger ECM molecule fragments, produced recombinantly, may represent a robust approach to increase activity, while still offering advantages over using the entire molecule (e.g. reduced antigenicity) (Culter and Garcia 2003). A key feature of natural ECM molecules is their susceptibility to cell triggered proteolysis, which permits cell invasion and subsequent remodelling of the matrix. Invasion and remodeling depend on the action of cell-secreted proteases that target specific sequences of the ECM molecules (Sternlicht 2001). Recent developments indicate that it is possible to synthesize cell-remodelable synthetic materials by utilizing these sequences as cross-linking agents (Lutolf et al. 2003 A).

Cell adhesion to synthetic surfaces is mediated by proteins, either adsorbed from the fluids placed in contact with the material, secreted by the cells, or placed at the surface. These ligands mediate the physical interaction between the material and receptors in the cell membrane. Polymer surfaces are frequently pretreated with a solution containing a purified protein or bioactive peptide to allow physical adsorption of the molecule and a specific cell-ligand interaction. This classical approach is the simplest technique for presenting proteins and peptides.

1.5.3 State of art of tested signals for stem cells recruitment

The exact mechanism by which MSC are mobilized into the circulation, undergo recruitment and transmigrate across the endothelium is not yet fully elucidated. However, it is probable that injured tissue expresses specific receptors or ligands to facilitate trafficking, adhesion and infiltration of MSC to the site of injury, similar to the recruitment of leukocytes to sites of inflammation (Sordi et al. 2009). Cytokines and chemokines are important factors in regulating mobilization, trafficking and homing of stem/progenitor cells (Liu et al. 2009).

Several studies aimed at investigating the different chemokine receptor profiles of human MSC and the chemotactic effect of particular cytokines on these cells have documented. Honczarenko et al. (2006) examined human bone marrow MSC for chemokine receptor and function and showed that the cells expressed a distinct set of chemokine receptors, namely: CCR1, CCR7, CCR9, CXCR4, CXCR5 and CXCR6. It was also demonstrated that chemokines corresponding to these surface receptors induced cellular responses e specific chemotaxis as well as b-actin filament reorganization

(CXCL12). Honczarenko et al. (2006) highlighted that these findings support the belief that certain chemokines, CXCL12 in particular, are important in bone marrowMSC homing and localization within the bone marrow, as has been determined for haematopoietic cells. In another study that was aimed at elucidating chemokine receptor expression on human bone marrow MSC and their role in mediating migration to tissues, Sordi et al. (2005) found that a small percentage of cells (2-25%) expressed a restricted set of chemokine receptors as well. However, though CXCR4, CXCR6, CCR1 and CCR7 were found to be expressed as well (consistent with the findings of Honczarenko et al.), receptors CCR9 and CXCR5 were not detected. Furthermore, cells were found to express CX3CR1 but thiswas not the case for the study by Honczarenko et al. Sordi et al. also showed that bone marrow MSC were capable of undergoing appreciable chemotactic migration in response to a restricted set of chemokines in vitro and that the attraction of these cells to an in vitro model of peripheral tissue was principally mediated by CX3CL1 and CXCL12.

Specifically, the CXC chemokine stromal derived factor-1 (SDF-1, also named CXCL12) has been associated with the migration, proliferation, differentiation and survival of several cell types such as human and murine haematopoietic stem and progenitor cells. CXCR4, the seven transmembrane G-protein coupled receptor of SDF-1, has been found to be exhibited by cell types including haematopoietic, endothelial, stromal and neuronal cells (Lapidot et al. 2002). Taken together, SDF-1 and CXCR4 have been found to have an important role in migration as indicated by studies on engraftment of bone haematopoietic stem/progenitor cells (Peled et al. 1999) as well as tumor

metastasis (Muller et al. 2001). The SD 1/CXCR4 axis also appears to regulate the migration of MSC. Using a transwell assay to investigate the response of the CXCR4 receptor to the ligand SDF-1,Wynn et al. (2004) observed the dose-dependent migration of human MSC to SDF-1 and concluded that the receptor contributes to MSC migration. To understand the growth factors/cytokines which can affect migration of MSC to injured tissues, Ozaki et al. (2007) compared the effects of 26 growth factors/cytokines on the migration activity of rabbit and human MSC using a microchemotaxis chamber. It was observed that the following consistently enhanced the migration of MSC at appropriate concentrations eplatelet-derived growth factor (PDGF)-BB, PDGF-AB, epidermal growth factor (EGF), HB-EGF, transforming growth factor (TGF-a), insulin growth factor (IGF-I), hepatocyte growth factor (HGF), fibroblast growth factor (FGF-2) and thrombin. In addition, as various combinations of these factors further enhanced the migration of MSC, it was suggested that combinations of growth factors may be important in eliciting the maximal chemotactic effect. In other studies, factors which have been shown to enhance the migratory capacity of MSC include IGF-1 (Li et al. 2007), matrix metalloproteinase 2 (MMP-2), membrane type 1 MMP (MT1-MMP) and tissue inhibitor of metalloproteinase 1 (TIMP-1) (Ries et al. 2007), galanin (Louridas et al. 2009), monocyte chemotactic protein-1 (MCP-1) (Dwyer et al. 2007, Wang et al. 2002.and monocyte chemotactic protein-3 (MCP-3) (Schenk et al. 2007).

Recently, it has been investigated the role of platelet-derived growth factor-B (PDGF-B)-activated fibroblasts in regulating recruitment, migration and differentiation of murine bone marrow MSCs in an in vitro wound healing

assay and a novel three-dimensional (3D) model, since local resting resident fibroblasts are activated after injury and play a critical role in recruiting MSCs. PDGF-B-activated fibroblasts caused significant increases in MSC migration speed compared to control as demonstrated by time-lapse photography in wound healing assay. Consistently, invasion/migration of MSCs into 3D collagen gels was enhanced in the presence of PDGF-B-activated fibroblasts. In addition, PDGF-B-aFBs induced differentiation of MSCs into myofibroblast. The regulatory effects of PDGF-Bactivated fibroblasts are likely to be mediated by basic fibroblast growth factor (bFGF) and epithelial neutrophil activating peptide-78 (ENA-78 or CXCL5) as protein array analysis indicated elevated levels of these two soluble factors in culture supernatant of PDGF-B activated fibroblasts. Blocking antibodies against bFGF and CXCL5 were able to inhibit both trafficking and differentiation of MSCs into 3D collagen gels while supplement of exogenous bFGF and/or CXCL5 promoted invasion/migration of MSCs into 3D collagen gels [Nedeau et al., 2008]. Our results reveal that PDGF-B-activated fibroblasts play a key role in the recruitment/migration and differentiation of MSCs and implicate a bFGF- and CXCL5- dependent mechanism in mediating these effects.

Activation by chemokines is an important step during recruitment of a reasonable number of progenitor cells to the damage tissues.

Recent studies determined the involvement of additional other chemokines. CXC-chemokines IL-8/Gro-α and its cellular receptors CXCR2 and CXCR1 contribute to homing of intravenous infused CD34+ progenitor cells to the ischemic myocardium (Kocher 2006). IL-8 is an inflammatory chemokine, which is able to stimulate angiogenesis (Muller et al.2002). Myocardial

infarction induces an increase of the expression of cardiac IL-8/Gro-α mRNA and increased serum concentrations of IL-8/Gro-α were associated with the number of CD133+ cells (Schomig et al. 2006). CD34+/CD117bright progenitor cells demonstrated a chemotactic response to IL-8 in vitro (Kocher 2006). Moreover, local injection of IL- 8 in the non-ischemic myocardium increased the recruitment of CD34+ cells (Kocher 2006) Neutralizing anti-IL-8/Gro-α-antibodies or antibodies against the IL-8 receptors, CXCR1 or CXCR2, reduced CD34+ cell-mediated improvement of neovascularization, establishing a role for CXC-chemokines (IL-8/Gro-α) for homing and neovascularization improvement by CD34+ cells.

In addition, blocking CXCR2 inhibited the incorporation of human EPCs expressing CXCR2 at sites of arterial injury (Grunewald 2007).

Furthermore, ischemia-induced VEGF acts as a chemoattractant to EPC (Kalka et al. 2000). Interestingly, VEGF is sufficient to induce the organ recruitment of bone-marrow-derived circulating myeloid cells and their perivascular localization via induction of SDF-1 expression by perivascular myofibroblasts, suggesting that different cytokines may cooperate during homing of bone marrow cells (Grunewald et al. 2006). In addition, invaded immune competent cells within the ischemic tissue may release further chemokines, such as MCP-1 or interleukins that can attract circulating progenitor cells (Fujiyama et al. 2003). Beside stimulating migration, MCP-1 and VEGF are capable of inducing the transendothelial migration of human ex vivo expanded myeloid EPC derived from peripheral blood in a β2-integrin-dependent manner in vitro (Chavakis et al. 2005). In accordance with these data, Spring et al. recently demonstrated the expression of the chemokine

receptors CCR2 and CCR5 in EPC and the expression of C–C chemokines in tumor vessels. In the same study, the inhibition of chemokine receptor signaling by PTX significantly reduced the incorporation of EPC in tumor vessels (Spring et al. 2005) supporting the involvement of Gprotein- coupled chemokine receptors in the homing process.

In conclusion, these data suggest that chemokines like IL-8, SDF-1 and probably others are involved in the trafficking of circulating pro-angiogenic cells and EPC from the bloodstream to ischemic tissues. Beside classical chemokines, other factors, which could be present in the ischemic myocardium, may also influence the recruitment of EPC. E.g., high mobility group box-1 (HMGB-1) is a nuclear protein, which is released extracellularly upon activation of cells by inflammatory cytokines and during cell necrosis and acts as a chemoattractant for inflammatory cells, stem cells and EPC in vitro and in vivo (Palumbo et al. 2004, Chavakis et al.2007). Since necrosis and inflammation are hallmarks of

ischemic and tumor tissues, it is conceivable, that HMGB-1 may be involved in the homing of EPC.

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CHAPTER 2

Mesenchymal Stem Cell migration in a 3Dcollagen model under the effects of growth factors/cytokines gradient

2.1 INTRODUCTION

Despite the enthusiasm, Tissue Engineered (TE) approaches have rarely found a massive and successful clinical implementation. In particular, cellular therapies are used mostly where an alternative conservative treatment cannot be pursued, thus slowing the transfer of TE products to healthcare systems and reducing their therapeutic impact. Additionally, international and local regulatory bodies can pose severe limitations on the use of exogenous/xenogenous cells and off-the-shelf availability is not at all straightforward. Indeed, the isolation of cells from patients, their amplification and subsequent *in vitro* culture are the most critical steps of the whole process, being highly costly and time consuming. For these reason, a large body of research is constantly focused on finding alternative and safer strategies which might eventually lead to a successful clinical implementation of TE products. One of the most promising strategy is the *in situ* tissue regeneration (Huang *et al.* 2002, Shantz *et al.* 2007, Zhao *et al.* 2008, Kimura *et al.* 2010). It basically aims at recapitulating those events occurring during the physiological processes of tissue repair and regeneration. In particular, in situ TE endeavours to recruit adult stem and progenitor cells that reside at the site of scaffold transplantation, providing the adequate stimuli to induce their differentiation and the consequent tissue regeneration, therefore bypassing the delicate procedures of cell isolation and *in vitro* culture. Whatever set of stimuli is chosen, cell recruitment is key central. The ideal scaffold should be "programmed" to broadcost specific biological signals with a predefined space and time distribution to selectively recruit target cells. Target cells do not necessarily reside far away from the

injured tissue: several works showed that mesenchymal progenitors are also circulating, although at low concentrations, in adult peripheral blood (Zvaifler *et al.* 2002). Moreover, Kuznetsov and colleagues (2001) demonstrated the presence of stem cells of extravascular mesodermal tissues in the circulating peripheral blood, capable to generate at least three phenotypes of the stromal system, namely osteoblasts, adipocytes, and reticular cells. The concentration of circulating stem cells significantly increases in stress/tissue injury conditions as demonstrated by Kucia *et al.* (2004). In fact, MSC have the ability to migrate from their niche to the site of tissue damage (Lu *et al.* 2001, Chen *et al.* 2001, Khaldoyanidi *et al.* 2008). However, the signals and their time and space presentation, required for stem cell homing and recruitment to injured sites are not thoroughly known. A variety of soluble factors like chemokines (Sordi *et al.* 2005, Honczarenko *et al.* 2006) and growth factors (Fiedler *et al.* 2002, Neuss *et al.*2004, Forte *et al.* 2006) are usually involved in the homing process. In particular, the CXC chemokine stromal cell–derived factor 1 (SDF-1 or CXCL12) is secreted by stromal cells from different tissues, such as bone marrow, lung, and liver (Bleul *et al.* 1996). Its chemotactic effect is mediated by the interaction with the chemokine receptor 4 (CXCR4) (Ma *et al.* 1998, Mason *et al.* 2001). Several studies reported different chemokine receptor profiles of human MSC and various chemotactic effects of SDF-1 on these cells (Stich *et al.* 2009, Son *et al.* 2006, Wynn *et al.* 2004, Abbott *et al.* 2004, Unzek 2007, Ringe *et al.* 2007). Among these, Sordi *et al.* (2005) showed that MSCs expressing the CXCR4 receptor are strongly chemoattracted by SDF-1 in a dose-dependent manner. Ryu *et al.* (2010) reported that SDF-1 participates in the activation

of those signal transduction pathways in MSCs that are involved in the regulation of directional cell migration. Concerning in situ tissue engineering, recent works focused on the incorporation/delivery of SDF-1 within/from scaffolds in order to recruit stem cells *in vivo*. In particular, Schantz *et al.* (2007) sequentially delivered VEGF, SDF-1 and BMP-6 in a PCL scaffold implanted in a rat model, demonstrating stem cell infiltration with evidences of angiogenesis and tissue precursor formation. Bladergroen *et al.* (2009) created an heparinized collagen scaffold loaded with SDF-1, subsequently implanted in a mouse model. They demonstrated that the release of SDF-1 was effective in recruiting hematopoietic stem cells. Thevenot *et al.* (2010) developed a system to constantly deliver SDF-1 within a PLGA scaffold implanted in a mouse model. Following this approach, they reported a significant recruitment of stem cells, with evidences on increased healing and angiogenesis and concomitant reductions of inflammation and scaffold encapsulation. As a whole, these works demonstrated the effectiveness of SDF-1 within a *in situ* TE scheme. However, the influence of SDF-1 gradient on the dynamics and regulation of stem cell migration within 3D scaffold, necessary for the design of cell-recruiting scaffold, is still missing. Therefore, in this work, we present a biomimetic experimental model to quantitatively analyze the effects of the SDF-1 gradients on the migratory parameters of MSC, in 3D collagen matrix. The setup may be simply reproduced and allows to study cell migration in a three dimensional environment.

2.2 Materials and methods

2.2.1 Cell cultures

Although MSCs attract much attention because of their pluripotency, this characteristic decreases during in vitro culture (Banfi et al.,Muraglia et al.,) using conventional two-dimensional (2D) culture conditions; MSC characteristics might be changed during culture and their therapeutic potential may be reduced. Previous studies have shown that during culture expansion MSC undergo an aging process in which their early progenitor properties, proliferation and homing capability are gradually lost (Banfi et al.; Digirolamo et al.; Prockop et al.).For these reason for the development of our experiment, as first step we used MG63 cells, an osteosarcoma lines, as "positive control". Indeed recent studies show the involvement of SDF-1/CXCR-4 system during osteosarcoma tumor progression (Perissinotto et al.) .

The MG63 osteosarcoma cell line (Franceschi *et al.*1985) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biowittaker, Walkersville, MD, USA), 2mM L-glutamine, 100 U/ml penicillin, and 0,1 mg/ml streptomycin (Sigma, St. Louis, MO, USA).

Human mesenchymal stem cells (hMSC) were purchased from Lonza Inc. (Walkersville, MD, USA). Three hMSC aliquots were pooled to reduce bias from samples derived from single donors, rinsed twice with PBS and plated at a final density of 2-5x10^6 cells/100 mm dish in Coon's modified Ham's F12 medium (Biochrom AG, Berlin, Germany), supplemented with 10% FBS and

1 ng/ml FGF-2 (LiStarFish, Milan, Italy) (1, 2). Cells were maintained in culture and used within their 3rd passaging (P1-P3).

For chemoinvasion assays hMSC were cultured in alpha Modified Eagle's medium (αMEM, Bio-Wittaker, Belgium) containing 10% (v/v) fetal bovine serum (FBS, HyClone, USA), 100 U/ml penicillin, 0,1 mg/ml streptomycin and 2mM L-glutamine (Sigma). All cultures were kept in a humidified atmosphere at 37°C and 5% of CO_2; the medium was replaced every 3 days. Whenever needed cells were trypsinized by standard protocols, washed in PBS and resuspended to the desired concentration for further use.

For cultures in 3D collagen lattices, hMSC were cultivated in reconstituted bovine collagen gel (APCOLL DEVRO) that was prepared following manufacturer's procedure. Briefly, 1 ml of 10X DMEM (Gibco, Life Technologies) was added to 8 ml collagen (stock solution: 3 mg/ml). Next, 1 ml of 0.1 M NaOH was added and pH 7.2 was reached by adding 0.1 M HCl. The resulting collagen solution (2.4 mg/ml), was gently mixed with the cells and allowed to gelify for approximately 40 minutes at 37°C, 5% CO_2.

2.2.2 Immunofluorescence analysis of cell surface antigen expression on hMSC or MG-63 cells.

The hMSC phenotype was assessed on P1 cells by immunofluorescence. Flow cytometric analysis was performed by staining $1x10^5$ cells/sample of each cell type at 4°C, for 30 minutes with the following monoclonal antibodies (moAb): fluoresceine isothyocyanate (FITC)-labeled anti-CD34, -CD45, (Miltenyi Biotech, Bergish-Gladbach, Germany), -CD105 (Acris Antibodies, Milan,

Italy), or R-phycoerithrin (PE)-labeled anti-CD63 (BD Biosciences, San Jose, CA, USA), -CD90 (BioSource Europe S.A., Nivelles, Belgium). FITC- and PE-labeled isotype-matched immunoglobulins (DakoCytomation, Glostrup, Denmark) were used as negative controls. Positivity for CD49a, CD117 and CXCR-4 was analyzed by indirect immunofluorescence; briefly $1x10^5$ cells of each sample were stained with the anti-CD49a or -CD117 monoclonal antibody (LiStarFish) or anti-CXCR4 (R&D System, Minneapolis, MN, USA) and incubated at 4° C for 30 min in the dark. Cells were rinsed twice with cold phosphate buffered saline (PBS) with 2% FBS, and were further stained with a secondary goat anti-mouse IgG-FITC moAb (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) and incubated for 30 min more at dark. Sample were rinsed twice with cold PBS+2% FBS, then cells were resuspended and analyzed by flow cytometry (FACScan, BD Biosciences, San Jose, CA). Some cell samples were permeabilized by exposure to a solution containing 0,1% Na-citrate and 0,1% Triton on ice for 5 minutes prior to immunostaining to evaluate the citoplasmic positivity. The effects of the three-dimensional environment on the expression of CXCR-4 in hMSC were evaluated by culturing the cells in collagen gels as described in the previous section. After 24 or 48h, collagen matrices were rinsed twice with PBS and digested by collagenase type A (2.5mg/ml in PBS for 40 minutes at 37C°; Roche Italia, Milan, Italy) to release cells from the matrix. The enzymatic digestion was neutralized with FBS and the cell population was collected by centrifugation for further processing.

2.2.3 mRNA extraction and Real Time RT-PCR

Cells aliquots, either from monolayer cultures on plastic dishes or from collagen matrices, at different passages and/or timings, were used for mRNA extraction, using the PerfectPure RNA Cultured Cell Extraction Kit (5-Prime GmbH, Hamburg, Germany),according to the manufacturer's instructions. Reverse transcriptions were performed by using the SuperScript™ III RT-PCR kit (Invitrogen, Milan, Italy). Aliquots of each cDNA sample were then processed for quantitative real time RT-PCR, using the RealMasterMix SYBR 2,5 (5-Prime) in an Eppendorf Mastercycler Realplex^2 apparatus; assessed genes were the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CXCR-4; primer couples were either derived from published literature (Giannoni *et al.* 2010) or purposely designed (see Supplemental Table 1). A denaturing step was performed at 95°C for 2 minutes; cycling conditions were set at 94°C for 30 sec, at 60°C for 30 sec and at 72°C for 30 sec, for 35 cycles. Real time PCR runs were performed in quadruplicate and the specificity of the reaction was counterchecked by the analysis of the melting curve of the amplified products.

The effects of the three-dimensional environment on the mRNA of CXCR-4 production in hMSC were evaluated by culturing and subsequent extraction of cells from collagen gels in analogous manner as described in the previous section. mRNA was then extracted in the same manner as the conventional 2D culture.

2.2.4 Chemoinvasion assay

Boyden Chambers (Neuro Probe Inc., Gaithersburg, MD USA) were used for the chemoinvasion assay to verify chemotactic effect of SDF-1 on MG63 and hMSC cells. MG63 and hMSC were cultured as previously described. Briefly, the upper and lower compartments of the chamber were separated by 8 μm pore-size polyvinylpyrrolidone-free polycarbonate filter (13-mm diameter Millipore). The lower compartment of chamber was loaded with αMEM serum free supplemented with 0.5% of bovine serum albumin (BSA; Sigma-Aldrich) only (control) or with SDF-1 at two different concentrations, i.e. 100 and 200 ng/ml. To determine whether cell invasion was effectively driven by SDF-1, the specific blocking agent for CXCR4, AMD3100 (Sigma-Aldrich) was loaded into the lower compartment of the chamber at the concentration 100 μg/ml, alongside SDF-1.

50 μl of Matrigel (BD Bioscience) was poured onto the membrane filters, in order to provide an adherent surface for the hMSC prior to their penetration toward chemotactic gradient. Then $5x10^4$ cells per chamber were preincubated in IMDM-0.5% BSA for 30 minutes and seeded onto the upper compartments and finally incubated at 37°C, 5% CO_2 for 4 hours. The lower surface of the filter was then fixed with para-formaldehyde and then stained with 1% crystal violet (Sigma). Digital images of the stained cells were collected under optical microscope at ×4 magnification. The fraction of the filter surface colonized by cells was determined using the National Institute of Health Image J free software (release 1,38X; http://rsb.info.nih.gov/ij/).

2.2.5 Chemotaxis experiment in three dimensional collagen gel

The analysis of cell movement under the effect of SDF-1 gradient was performed in a similar way to that presented by Knapp et al. (1999). Briefly, two rectangular shaped wells (270 and 540 μl volume) were punched in a 3 mm thick Polydimethylsiloxane (PDMS) cylinder (figure.1). The two wells were separated by a 2 mm large wall with a 2 mm channel in the middle. The chamber was attached on a glass coverslide using a silicone glue and autoclaved prior cell seeding.

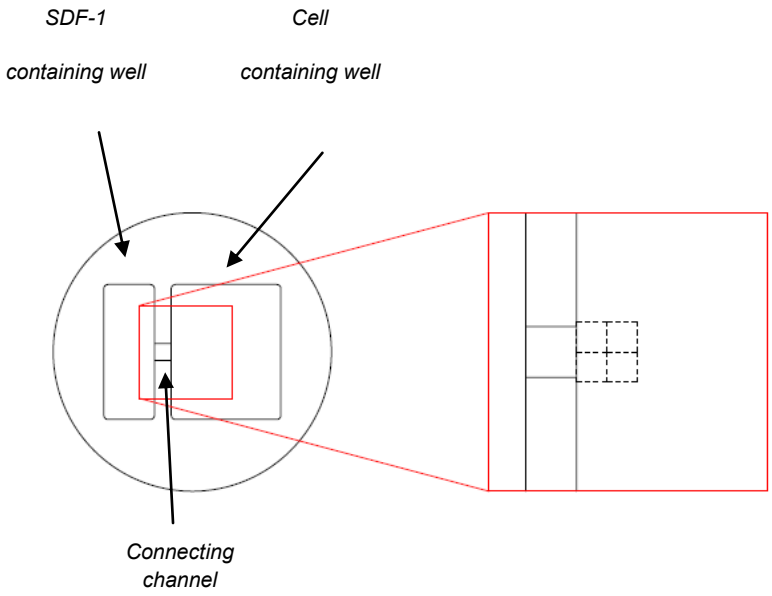

Figure 1. Schematic of the chamber used for chemotaxis experiments. The dashed lines in the enlargement on the right hand side mark off the regions that were observed during the experiment.

The chemotaxis experiment were carried out by injecting a cell-seeded collagen solution ($15x10^3$ MG63 or hMSC in a 2.4 mg/ml collagen solution) in the larger well. The gel was incubated at 37°C, 5% CO_2. After 24 h, a 2.4

mg/ml collagen solution, either doped with 0, 30 or 60 ng of SDF-1, was injected in the small well. The same solution was carefully injected within the channel that connects the two wells in order to allow the diffusion of the factor. The solution was allowed to gelify for approximately 1 h in a mini-incubator mounted on the automated stage of an inverted optical microscope. According to the concentration of SDF-1 used, the experiment will be referred as 'control', '$-12\,\mathrm{ng\cdot ml^{-1}\cdot mm^{-1}}$' or '$-25\,\mathrm{ng\cdot ml^{-1}\cdot mm^{-1}}$', respectively. Videos of migrating cells were obtained by acquiring images (1.6 × 1.2 mm) on selected regions of the cell populated collagen gel (the regions under investigation are highlighted in figure 1), every 10 minutes for 24 hours using an Olympus IX 50 optical microscope (Olympus Co., Tokio, Japan) equipped with a mini – incubator mounted on an automated stage (PRIOR, Rockland, MA) and a CoolSnap Camera (Photometrics, Tucson, AZ). The images were focused on three different planes: approximately 0.5 mm above the bottom of the channel, middle plane of the channel and approximately 0.5 mm above the middle plane, thus resulting in 12 time lapse videos. In each experiment 20 – 35 cells were focused.

2.2.6 Simulation of the temporal evolution of the SDF-1 concentration gradient

A finite element method (FEM) based approach was used to predict SDF-1 diffusion through the collagen gel. Diffusion was assumed to be governed by the diffusion equation $\frac{\partial c}{\partial t} + \nabla \cdot (D\nabla c) = 0$, where, c denotes the concentration of SDF-1 and D the diffusion coefficient of the factor in 2.4 mg/ml collagen gel

and ∇ is the standard nabla operator. The model was specified by using a SDF-1 concentration of 111 ng/ml or 222 ng/ml to simulate the LG or HG conditions respectively. The diffusion coefficient was assumed to be 10^{-6} cm^2/s. Such a value was taken from literature data, reporting the diffusion coefficient of model molecules, similar in dimensions as SDF-1, diffusing in analogous media to the one we used (Saadi *et al.* 2007; Nauman *et al.* 2007). The model was implemented in COMSOL Multiphysics 3.5 (Comsol Inc., Burlington, MA) and solved as time dependent problem. Factor diffusion was evaluated on the middle plane of the chemotaxis chamber, as reported in figure 1a. The 2D domain was discretized using predefined triangular mesh. Computations were done with the UMFPACK direct solver as linear system solver on a PC with an Intel Quad Core 2.4 GHz CPU (Intel Co., Santa Clara, CA) running Windows (Microsoft, Redmond, WA).

2.2.7 Evaluation of the cell tracking parameters

Cell trajectories, i.e. the sequence of cell centroids coordinates, were reconstructed from time lapse video using Meramorph software. Bias speed of the cell population along the gradient, S_{Bias}, and root mean squared speed, S_{Random}, were chosen as the representative parameters for describing cell migration. S_{Bias} is estimated by plotting the average position of the cell population along the gradient direction, i.e. x axis, against time. A linear drift is observed in the case of anisotropic environments, therefore S_{Bias} is evaluated by fitting the average position of the cell population with a straight line. The average position in the direction orthogonal to the gradient remains

unchanged. S_{Random}, which provides information on the random component of speed, is evaluated by fitting the cell's mean squared displacement with a persistent random walk model. In more details, mean squared displacement was evaluated using overlapping time intervals (Dickinson and Tranquillo (1993). In the case of non-zero S_{Bias}, linear drift was subtracted from cell displacement, in order to take into account the random displacements only. The persistent random walk chosen for the data fitting was the one presented by Kipper *et al.* (2007).

Chemotaxis index, CI, is defined as the ratio of the trajectory contour length with the head-to-tail vector length. Statistical significance among S_{Bias} values was assessed by ANOVA test performed in Matlab. Statistical significance among S_{Random} or CI values was assessed by performing a non-parametric Kruskal-Wallis test in Matlab. p values < 0.05 were considered significant.

2.3 RESULTS

2.3.1 Characteristics of hMSC derived from bone marrow

Human MSC were expanded in monolayer cultures and phenotyped by FACS analysis, upon detachment from plasticware and immediately prior to use in collagen matrices. Cells displayed a standard fibroblastic morphology; their phenotypic profile comprised positivity to CD49a, CD63, CD90 and CD105 for more than 90% of the assessed cells. At the same time less than 2% of the total cell population resulted positive for classical hematopoietic markers, such as CD34, CD45 or CD117. Interestingly, upon culturing, over than half of the total cell number scored positive for the expression of CD146, a

relevant marker for the identification of osteogenic precursors able to regenerate also a complete and functional marrow (Sacchetti *et al.* 2007). Surface expression of the SDF-1 receptor, CXCR4, instead, was established for 16% of the assessed cells, although single-staining procedures did not allow us to establish the percentage of cells contemporarily positive for CXCR4 and CD146. Nonetheless, as a whole, the available cells displayed a classical phenotypic signature of mesenchymal precursors; they were thus retained representative of the population of stromal stem progenitor cells, either residing within or able to migrate to lesion sites, and involved in the regeneration of damaged mesenchymal tissues.

2.3.2 Expression of CXCR4 receptor in MG63 and hMSC

Real Time PCR analysis showed that transcripts for CXCR4 are highly expressed in MG63 cells; human MSC express the receptor mRNA as well, although at lower levels, as reported in figure 2.

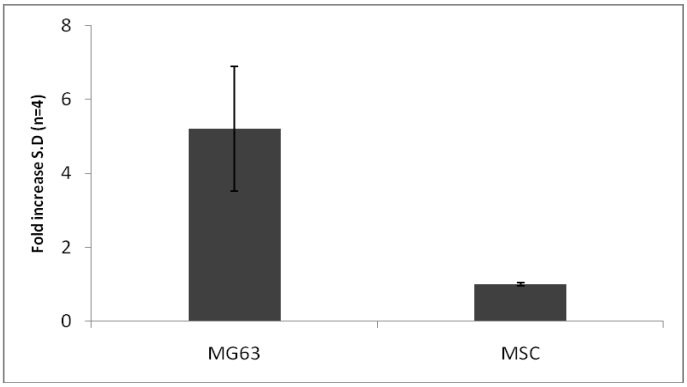

Figure 2. Relative expression levels for CXCR-4 mRNA in MG63 and BMSC cells, as assessed by Real time RT PCR.

Transcript data was paralleled by detailed cytofluorimetric analysis, to discriminate surface receptor expression from intracellular availability of the protein. In more details, MG63 showed high levels of intracellular expression of the receptor (~ 64% of the cell population resulted positive at FACS analysis), while its surface expression was detected in approximately 43% of the assessed cells. On the other hand, 93% of hMSC were positive for intracellular expression of the receptor, whereas only 16% of the stem cells displayed the receptor on the membrane surface, as already indicated (Table 1).

	INTRACELLULAR	CELL MEMBRANE
MG63	63.9%	42.9%
MSC	93.0%	15.9%

Table 1 Flow cytometric analysis of CXCR-4 expression in MG63 and BMSC cells. The percentage of positive cells for the expression of receptor was evaluated both intracellulary and on cell membrane.

Relevantly, in hMSC, CXCR4 mRNA levels are strongly affected by cell passaging, as shown in Table 2. Surface expression of CXCR4 is affected by stem cell passage as well, i.e. a fourfold decrease in expression is observed from passage 2 to passage 4 (Table 2).

	RT PCR	FACS
P2	1.000 ± 0.035	15.9%
P4	0.016 ± 0.001	4.0%

Table 2 Expression of CXCR-4 in BMSC at different passages. Results show that stem cells passage affects both mRNA production and the expression on cell membrane of CXCR-4 receptor.

Surprisingly, short-term (24-48 h) culture in tridimensional collagen matrix resulted in up-regulation of cell surface expression of CXCR4 (figure 3), calling for the existence of a mechanism of either transcriptional regulation and/or CXCR4 receptor presentation quite sensitive to the cell 2D-versus-3D microenvironment status, possibly depending on tensegrity (Ingber 2008; RayChaudhury *et al.* 2001).

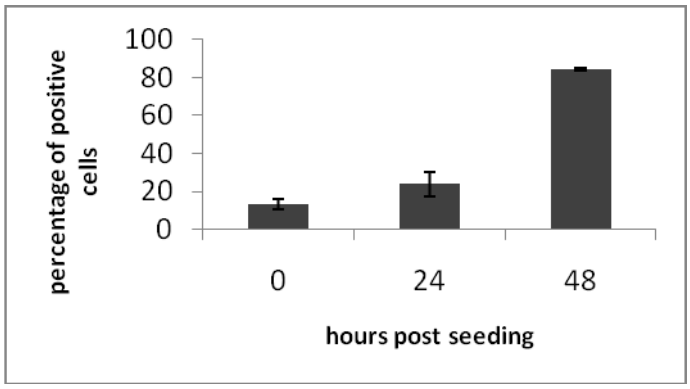

Figure 3. Time-dependent variations in the percentage of CXCR4-positive BMSC, as assessed by cytofluorimetric analysis, upon seeding and culturing cells in 3D collagen gels.

2.3.3 Chemoinvasion assay

Results of the Boyden Chamber experiments are reported in terms of the fraction of the filter area occupied by cells. MG63 extensively migrate through

the filter, irrespective of the concentration of the gradient that is loaded (figure 4). In both cases, either 100 ng/ml or 200 ng/ml, more than 75% of the filter is colonized by the cells. Invasion is halted in presence of the receptor blocking agent AMD3100, being similar to the control experiment. Conversely, hMSC respond in a less effective manner. In particular, just 8% of the filter is occupied by the cells when the chamber is loaded with 200 ng/ml of SDF-1 (figure 4). Also for hMSC, in the presence of the AMD3100 the infiltration of cells does not differ from the control experiment (figure 4).

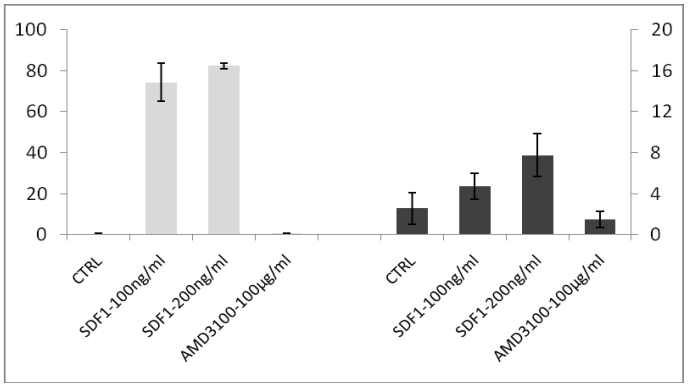

Figure 4. Chemoinvasion Assay of SDF-1 on MG63 (light grey) and hMSC cells (dark grey). Different concentrations of SDF-1 (0, 100 and 200 ng/ml) were tested. Percentage of the filter surface occupied by MG63 or hMSC is reported on the left or right axis, respectively.

2.3.4 SDF-1 concentration gradient profile

The SDF-1 distribution obtained along the gradient axis of the two-dimensional middle plane is shown in figure 5. In particular, the figure highlights the concentration profile in the region interested in cell tracking, i.e. in the region spanning approximately 2 mm from the opening that connects the two wells of the chemotaxis chamber. The gradient is established in the first hours of the experiment and is present during the

whole cell tracking experiment, in particular it is reasonably stable from hour 12 to 24, i.e. assuming that the gradient is linear, a 12% change is observed in gradient slope, in the 12 – 24 h interval. The concentration of SDF-1 the cells are exposed to is not different to the values that have previously reported in literature which are known to elicit a chemotactic response (Schantz *et al.* 2007, Son *et al.* 2009).

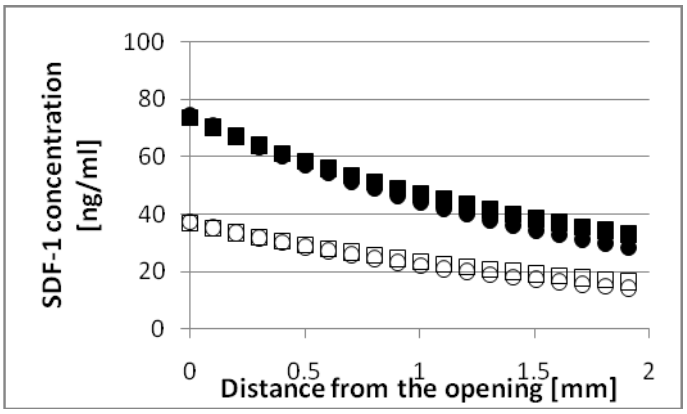

Figure 5. Simulated SDF-1 concentration profiles. The profiles were calculated at three time points namely 3h (triangles), 12 h (circles) and 24 h (squares). Hollow symbols refer to $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ conditions, solid symbols refer to $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ conditions.

2.3.5 Cell migration in 3D isotropic and anisotropic collagen gel

Windrose plots of the MG63 trajectories are reported in figure 6. In the absence of SDF-1, cells move randomly (figure 6A), whereas in presence of the SDF-1 gradient MG63 predominantly migrate toward the chemoattractant source (figure 6B and 6C). Most of the cell tracks are co-aligned with the horizontal axis, i.e. the direction of SDF-1 gradient,

particularly in the $-25\,ng\cdot ml^{-1}\cdot mm^{-1}$ conditions. Moreover, the SDF-1 gradient affects cells directional persistence. In fact, MG63 tracks are highly scattered in the control, whereas they are smoother when the gradient is present.

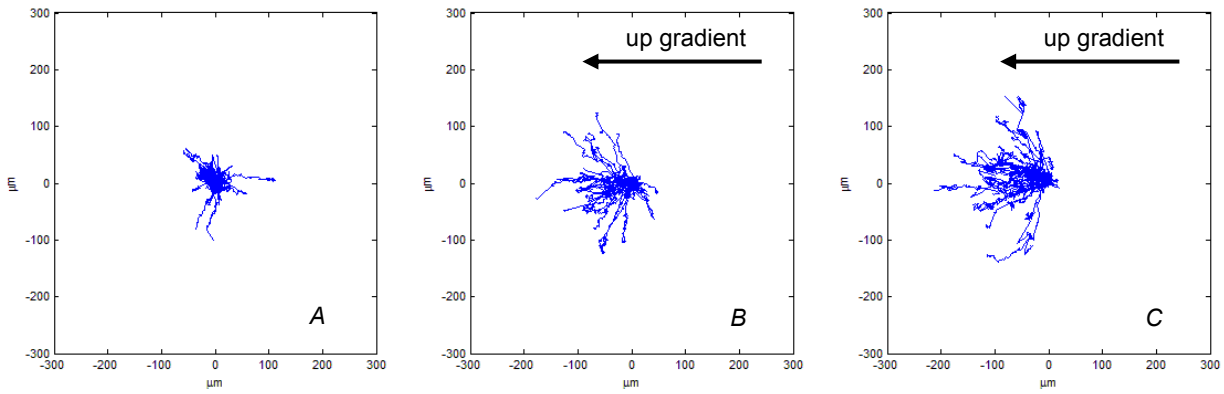

Figure 6. Windrose plots of MG63 trajectories recorded in the 24 h cell tracking experiment. A. Control experiment; B. $-12\,ng\cdot ml^{-1}\cdot mm^{-1}$ condition experiment; C. $-25\,ng\cdot ml^{-1}\cdot mm^{-1}$ condition experiment. SDF-1 factor diffuse from the left to the right

The CI value of the control experiment is significantly lower than the CI of both $-12\,ng\cdot ml^{-1}\cdot mm^{-1}$ and $-25\,ng\cdot ml^{-1}\cdot mm^{-1}$, however, no significant difference is observed between the latter (figure 7A). The effects of SDF-1 gradient on the migration speed values are indicated in figure 7B and 7C for the values of S_{random} and S_{bias} respectively. Although the average value of S_{random} increases with the concentration gradient slope, no significant differences are observed among S_{random} values. On the other hand, a significant difference of S_{bias} is observed among the three experimental setups.

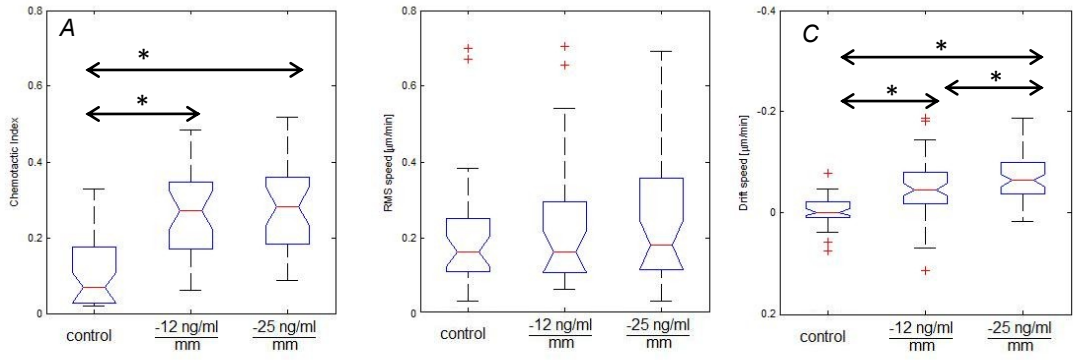

Figure 7. Box and whiskers plots of the migration parameters, evaluated for MG63 cells in the control, $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ and $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ experimental conditions. A. Chemotaxis Index; B. RMS speed; C. Drift speed. + symbols represent outliers. Significant differences are marked with asterisks

Human MSCs migrate randomly within SDF-1 free collagen matrices, as depicted in figure 8A, whereas they migrate mostly toward the SDF-1 source in the $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ (figure 8B) and $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ experiments (figure 8C). Moreover time lapse videos of hMSC in the absence of factor, showed that the cells moved predominantly in a "back-and-forth" fashion. In presence of a chemoattractant source, however, hMSC moved more persistently.

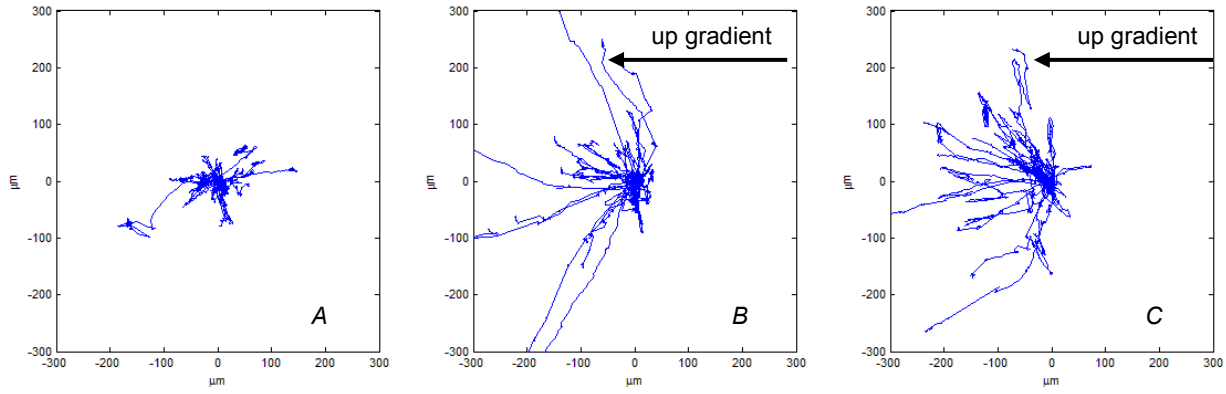

Figure 8. Windrose plots of hMSC trajectories recorded in the 24 h cell tracking experiment. A. Control experiment; B. $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ condition experiment; C. $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ condition experiment. SDF-1 factor diffuse from the left to the right

This evidence is shown in figure 9A in which the CI of the three experimental setups are reported. In particular, the CI value of control experiment is significantly lower than the CI of both $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ and $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$. However, the concentration of SDF-1 factor does not induce differences between the CI of the two experiments. In this case however, SDF-1 significantly affects random speed, as depicted in figure 9B. S_{random} values of both $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ and $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ conditions are significantly higher with respect to the control however, no difference was observed between them. Bias speed values are also dramatically affected by the chemotactic source. In particular S_{bias} of both $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ and $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ are significantly higher than the control, but the values do not differ from each other (figure 9C).

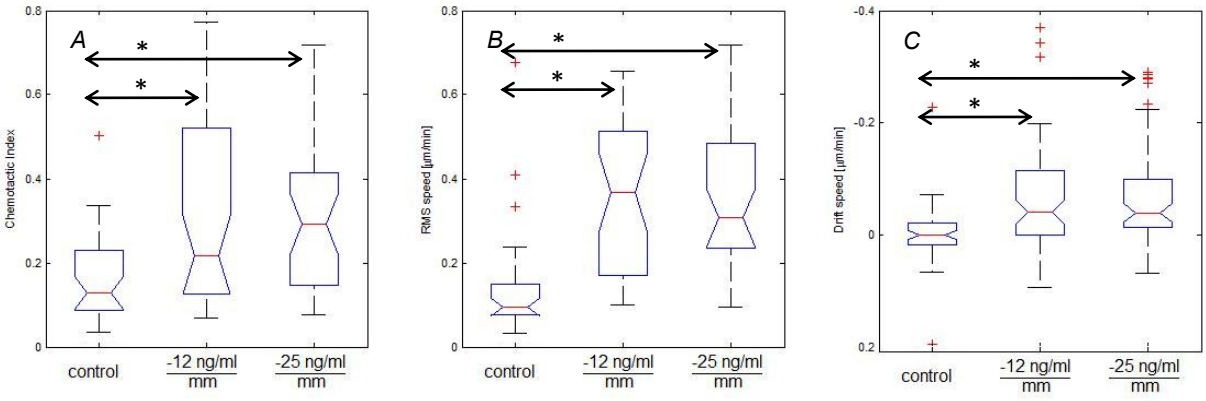

Figure 9. Box and whiskers plots of the migration parameters, evaluated for hMSC cells in the control, $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ and $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ experimental conditions. A. Chemotaxis Index; B. RMS speed; C. Drift speed. + symbols represent outliers. Significant differences are marked with asterisks.

2.4 DISCUSSION

Regenerative medicine and TE hold the promise to solve several problems related to tissue and organ replacements. They are based on the sapient combination of cells, material scaffolds and *in vitro* culturing conditions, all of which aim at generating a hybrid biological/synthetic device to substitute the functions of an injured or diseased tissue or organ. MSCs are increasingly gain attention as cell source for TE applications, due to their characteristics of self-renewal and multipotency. In fact, several works reported the possibility to induce hMSC differentiation under specific microenvironmental conditions, giving rise to muscle, brain, liver, cartilage, bone, fat and blood vessels (Jiang *et al.* 2002., Pittenger *et al.* 2004). However, Yet, a considerable number of MSC characteristics have to be studied in order to develop effective therapeutic strategies for their use in TE. A cascade of signals orchestrates homing and recruitment processes of stem cells in vivo. However, the migratory behavior of MSC under the effects of such signals is not fully explored. In this work we developed an experimental model that allows to gain a better insight into the dynamics of cell migration in a three dimensional biomimetic environment. In particular, we used a 3D collagen lattice in which a gradient of SDF-1 is established. In order to optimize the experimental setup in terms of chamber dimension, SDF-1 concentration and experiment time length, we have used the MG63 osteosarcoma cell line. These cells share certain similarities with MSCs (Perissinnotto *et al.* 2005), but their behaviour under the effect of SDF-1 is not affected by cell passage or donor source. In contrast, since MSC are primary cells, one invariably deals with a relevant heterogeneity in terms of

cell behavior, in particular migratory behaviour. Such an heterogeneity also depends on cell passage, which brings in another source of variability. In order to evaluate SDF-1 recruiting ability and to assess possible dose dependencies, we performed a conventional Boyden Chamber experiment with MG63. The results depicted in figure 4 show that SDF-1 has chemotactic activity on MG63. Although the average values of the invading cells increases as the SDF-1 concentration increases, a significant difference between the two values is not evident. Human MSC exhibit a similar behavior in the same experimental conditions, although only small number of stem cells migrate toward the factor, compared to the MG63 (figure 4). This result is consistent with real-time RT PCR expression and cytofluorimetric data: higher levels of the CXCR4 receptor, either intracellular or available on the cell surface, are expressed by the osteosarcoma line, with respect to hMSC. Consequently MG63 cells display a wider reactivity to the ligand-induced chemotactic stimulus. Clearly, boyden chamber experiments provide only basic information on the chemotactic effect of specific factors. To gain a better insight into the dynamics of cell migration under the effect of SDF-1, we performed a time lapse video microscopy of cells moving within 3D lattices. In particular, the experiments were performed in reconstituted collagen gels since they mimic more closely the in vivo environment rather than the 2D Boyden Chambers. Moreover, it has been already reported that it is possible to easily sustain spatial gradients of bioactive molecules within these sort of gels (Knapp et al. 1999). The chemotaxis experiments on MG63 show that SDF-1 mainly affects the directional persistence of the cells rather than the random component of their speed. This is evident from figure 7A

and figure 7C: the average position of the cell population along the gradient axis has a non zero linear drift in both $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ and $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ experiments and such drift speed significantly increases with the gradient slope. Accordingly, CI also reflects the effects of the SDF-1 factor. In fact, in the case of an isotropic environment as the control, CI is 0.1 which approximates a pure random movement, whereas in the presence of SDF-1, either in $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ or $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ conditions, CI is close to 0.3 implying a more persistent motion. Random root mean squares values do not change significantly in the three experimental conditions suggesting that the presence of the factor does not affect the random motility of the cell. Presumably not affecting the frequency with which the cytoskeleton machinery exerts its function.

The migratory behaviour of hMSC is dramatically affected by the presence of the chemotactic factor, as evidenced by the shape of the trajectories and the increase of both drift speed and CI. The reactivity of hMSC to SDF-1 might be apparently in contrast with the results of the Boyden Chamber experiments and FACS data, where a low expression of CXCR4 was evidenced. However these data refer to stem cells cultured in a two dimensional environment: culture in collagen lattice is sufficient per se in stimulating more cells to express the receptor at the membrane level (figure 3), which consequently sustains an increased cells' responsiveness to the factor compared to a 2D case. Although a non zero drift speed toward the factor was assessed in both $-12\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ or $-25\,\mathrm{ng}\cdot\mathrm{ml}^{-1}\cdot\mathrm{mm}^{-1}$ condition, the trajectories of the hMSC are more scattered around the gradient direction compared to those of the MG63. Of course hMSC are primary cells, therefore possessing an intrinsic

biologic variability in terms of motility. Moreover, the hMSC that were used for the chemotactic experiments were pooled from three different donors, which brings in another source of variability. Nevertheless, the directional motion is evident and statistically significant variations in the migration parameters were assessed. Differently from the MG63 case though, SDF-1 affects the random component of the MSC speed, i.e. S_{random} increases of a factor 2.3 when SDF-1 is present (figure 9B). Ryu *et al.* (2010) demonstrated that SDF-1 participates in the activation of transduction pathways which are known to regulate actin machinery and directional motion. Our data seem to confirm this evidence as demonstrated by an enhanced migratory behaviour of hMSC under the effects of SDF-1.

Taken together these data demonstrate that SDF-1 explicates a strong chemotactic effect within three dimensional collagen lattices. The event is particularly enhanced for hMSC, affecting different aspects of cellular motility, namely the shape of the paths, the biased component and the random component of the speed. Few issues might be arisen on the experimental procedure that has been presented. The spatial concentration gradient of the factor was not characterized, along with its evolution with time. This involves an uncertainty associated with the SDF-1 concentration the cells are exposed to. However the FEM simulations show that the gradient in the two experimental conditions is sufficiently stable during the 24 hours of the migration experiment. Moreover, hMSC are known to produce SDF-1 when they are exposed to the same factor (Stich *et al.* 2009). This, of course, induces local fluctuations of SDF-1 concentration which, macroscopically might alter the concentration gradients profile. This

occurrence might be difficult to quantify along with the migration experiment. Nevertheless, both MG63 and hMSC unequivocally perceive the presence of the chemotactic factor, as demonstrated by the cell trajectories and the drift speed. The uncertainty that is associated to the cell position with respect to the concentration gradient is reflected in the large standard deviations that we have observed when evaluating the migration parameters. Nevertheless, statistically significant differences were found in the drift speed, CI and random speed. In conclusion, the present work represent a first step toward the characterization of the migratory behavior of hMSC exposed to SDF-1. In conclusion, this work quantifies relevant migration parameters of hMSC subjected to a SDF-1 gradient. The results may provide useful cues for the design and production of smart scaffold that are able to recruit in vivo stem cell.

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CHAPETER 3

Effect of SDF-1 pre-stimulation on MSC migratory behaviour

3.1 Introduction

Critical to the implementation of Mesenchymal Stem Cells (MSCs) in TE strategies is a thorough understanding of which external signals in the stem cell microenvironment provide cues to control their fate decision in terms of proliferation, migration or differentiation into a desired, specific phenotype. The precise spatial and temporal presentation of factors directing stem cell behaviour is extremely important during development and natural healing events, and it is possible that this level of control will be vital to the success of many regenerative therapies.

The efficiency of therapies to augment recovery from damaged tissues depends on not only sufficient amount of MSCs, but also efficient delivery of these cells to the desired target tissue. Recruitment of bone-marrow derived MSC to repair damaged tissues and regeneration of the tissue is a complex multi-step process. It involves sensing the signal from the remote injured tissue that calls for the release of MSCs from their storage niche into circulation, homing of circulating MSCs to the target tissues, and in situ proliferation and differentation of MSCs into matured functional cells. The release of MSCs from their niche in the bone marrow into circulation is known as mobilization. The molecular mechanisms for mobilization of MSCs are poorly understood. Knowledge about the nature of signals released from the injured tissue to mobilize MSCs in the bone marrow is also very limited. One hypothesis is that cytokines that are up-regulated under injured conditions are released into circulation from remote tissues, stimulating MSC to down-regulate the adhesion molecules that hold them at their niche. (Zhang et al. 2008).

Cytokines and chemokines play critical roles in regulating mobilization, trafficking and homing of stem/progenitor cells (Wang et al. 2002).

In the previous chapter we demonstrated the influence of SDF-1 gradient on the dynamics of hMSC migration in 3D collagen matrix. We showed that hMSC, cultured in 3D collagen matrix are strongly chemoattracted by SDF-1 gradient.

The critical role of SDF-1/CXCR-4 axis in modulating mobilization of MSCs is demonstrated by several studies. Zhang and colleagues (2008) showed that over-expression of CXCR-4 on MSCs surface augments myoangiogenesis in the infracted myocardium.

Tissue regeneration requires transfer of a vast amount of information between different cell populations and organ systems, and growth factors play an important role in this communication and information transfer between cells and their microenvironment and between organ systems. Growth factors and chemokines transmit signals regulating development and normal growth control, including the stimulation or inhibition of cellular proliferation, differentiation, migration, adhesion, and gene expression (Alberts 2008). Growth factors and chemokines initiate their action by binding to specific receptors on the surface of target cells, and the level of expression of these receptors, in part, determines cell responsiveness. The chemical identity, concentration, duration, and context (e.g., presence and sequence of multiple factors) of these molecules contain information that dictates cell fate.

Gradients of chemokines play key role in both tissue regeneration and developmental biology, as they provide a mechanism by which cells can obtain spatial and directional cues.

3.2 Materials and methods

3.2.1 Cell cultures

Human mesenchymal stem cells (hMSC) were purchased from Lonza Inc. (Walkersville, MD, USA). Three hMSC aliquots were pooled to reduce bias from samples derived from single donors, rinsed twice with PBS and plated at a final density of 2-5x10^6 cells/100 mm dish in Coon's modified Ham's F12 medium (Biochrom AG, Berlin, Germany), supplemented with 10% FBS and 1 ng/ml FGF-2 (LiStarFish, Milan, Italy) (1, 2).

For migration assays hMSC were cultured in alpha Modified Eagle's medium (αMEM, Bio-Wittaker, Belgium) containing 10% (v/v) fetal bovine serum (FBS, HyClone, USA), 100 U/ml penicillin, 0,1 mg/ml streptomycin and 2mM L-glutamine (Sigma). All cultures were kept in a humidified atmosphere at 37°C and 5% of CO_2; the medium was replaced every 3 days. Whenever needed cells were trypsinized by standard protocols, washed in PBS and resuspended to the desired concentration for further use.

For cultures in 3D collagen lattices, hMSC were cultivated in reconstituted bovine collagen gel (APCOLL DEVRO) that was prepared following manufacturer's procedure. Briefly, 1 ml of 10X DMEM (Gibco, Life Technologies) was added to 8 ml collagen (stock solution: 3 mg/ml). Next, 1 ml of 0.1 M NaOH was added and pH 7.2 was reached by adding 0.1 M HCl.

The resulting collagen solution (2.4 mg/ml), was gently mixed with the cells and allowed to gelify for approximately 40 minutes at 37°C, 5% CO_2.

3.2.2 Immunofluorescence analysis of cell surface antigen expression on hMSC.

The hMSC phenotype was assessed on P1 cells by immunofluorescence. Flow cytometric analysis was performed by staining $1x10^5$ cells/sample of each cell type at 4°C, for 30 minutes with the following monoclonal antibodies (moAb): fluoresceine isothyocyanate (FITC)-labeled anti-CD34, -CD45, (Miltenyi Biotech, Bergish-Gladbach, Germany), -CD105 (Acris Antibodies, Milan, Italy), or R-phycoerithrin (PE)-labeled anti-CD63 (BD Biosciences, San Jose, CA, USA), -CD90 (BioSource Europe S.A., Nivelles, Belgium). FITC- and PE-labeled isotype-matched immunoglobulins (DakoCytomation, Glostrup, Denmark) were used as negative controls. Positivity for CD49a, CD117 and CXCR-4 was analyzed by indirect immunofluorescence; briefly $1x10^5$ cells of each sample were stained with the anti-CD49a or -CD117 monoclonal antibody (LiStarFish) or anti-CXCR4 (R&D System, Minneapolis, MN, USA) and incubated at 4° C for 30 min in the dark. Cells were rinsed twice with cold phosphate buffered saline (PBS) with 2% FBS, and were further stained with a secondary goat anti-mouse IgG-FITC moAb (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) and incubated for 30 min more at dark. Sample were rinsed twice with cold PBS+2% FBS, then cells were resuspended and analyzed by flow cytometry (FACScan, BD Biosciences, San Jose, CA). Some cell samples were permeabilized by exposure to a solution containing 0,1% Na-citrate and 0,1% Triton on ice for 5 minutes prior to

immunostaining to evaluate the citoplasmic positivity. The effects of the SDF-1 prestimulation on the expression of CXCR-4 in hMSC were evaluated by culturing the cells in collagen gels as described in the previous section. After 24, collagen matrices were rinsed twice with PBS and digested by collagenase type A (2.5mg/ml in PBS for 40 minutes at 37C°; Roche Italia, Milan, Italy) to release cells from the matrix. The enzymatic digestion was neutralized with FBS and the cell population was collected by centrifugation for further processing.

3.2.3 mRNA extraction and Real Time RT-PCR

Cells aliquots, either from monolayer cultures on plastic dishes or from collagen matrices, at different passages and/or timings, were used for mRNA extraction, using the PerfectPure RNA Cultured Cell Extraction Kit (5-Prime GmbH, Hamburg, Germany),according to the manufacturer's instructions. Reverse transcriptions were performed by using the SuperScript™ III RT-PCR kit (Invitrogen, Milan, Italy). Aliquots of each cDNA sample were then processed for quantitative real time RT-PCR, using the RealMasterMix SYBR 2,5 (5-Prime) in an Eppendorf Mastercycler Realplex^2 apparatus; assessed genes were the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CXCR-4; primer couples were either derived from published literature (Giannoni *et al.* 2010) or purposely designed. A denaturing step was performed at 95°C for 2 minutes; cycling conditions were set at 94°C for 30 sec, at 60°C for 30 sec and at 72°C for 30 sec, for 35 cycles. Real time PCR runs were performed in quadruplicate and the specificity of the reaction was

counterchecked by the analysis of the melting curve of the amplified products.

The effects of the prestimulation with SDF-1 on the mRNA of CXCR-4 production in hMSC were evaluated by culturing and subsequent extraction of cells from collagen gels in analogous manner as described in the previous section. mRNA was then extracted in the same manner as the conventional 2D culture.

3.2.4 Chemotaxis experiment in three dimensional collagen gel

The analysis of cell movement under the effect of SDF-1 gradient was performed in a similar way to that presented by Knapp et al. (1999). Briefly, two rectangular shaped wells (270 and 540 μl volume) were punched in a 3 mm thick Polydimethylsiloxane (PDMS) cylinder (same device described in the previous chapter). The two wells were separated by a 2 mm large wall with a 2 mm channel in the middle. The chamber was attached on a glass coverslide using a silicone glue and autoclaved prior cell seeding.

The chemotaxis experiment were carried out by injecting a cell-seeded collagen solution ($15x10^3$ hMSC in a 2.4 mg/ml collagen solution),doped with SDF-1 at two different concentration (100 or 200 ng/ml) in the larger well. The gel was incubated at 37°C, 5% CO_2. After 24 h, a 2.4 mg/ml collagen solution, either doped with 0, 30 or 60 ng of SDF-1, was injected in the small well. The same solution was carefully injected within the channel that connects the two wells in order to allow the diffusion of the factor. The solution was allowed to gelify for approximately 1 h in a mini-incubator mounted on the automated stage of an inverted optical microscope. Videos of

migrating cells were obtained by acquiring images (1.6 × 1.2 mm) on selected regions of the cell populated collagen gel (the regions under investigation are highlighted in figure 1), every 10 minutes for 24 hours using an Olympus IX 50 optical microscope (Olympus Co., Tokio, Japan) equipped with a mini – incubator mounted on an automated stage (PRIOR, Rockland, MA) and a CoolSnap Camera (Photometrics, Tucson, AZ). The images were focused on three different planes: approximately 0.5 mm above the bottom of the channel, middle plane of the channel and approximately 0.5 mm above the middle plane, thus resulting in 12 time lapse videos. In each experiment 20 – 35 cells were focused.

3.3 RESULTS

3.3.1 Characteristics of hMSC derived from bone marrow

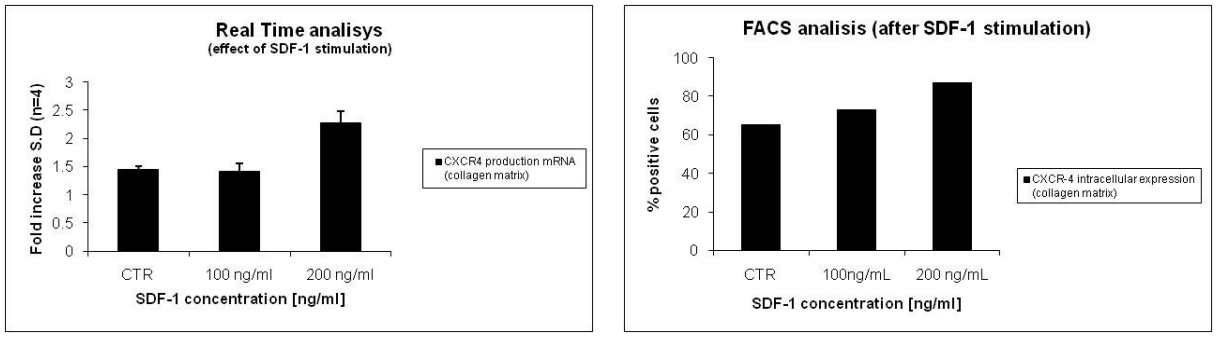
Human MSC were expanded in monolayer cultures and phenotyped by FACS analysis, upon detachment from plasticware and immediately prior to use in collagen matrices. Cells displayed a standard fibroblastic morphology; their phenotypic profile comprised positivity to CD49a, CD63, CD90 and CD105 for more than 90% of the assessed cells. At the same time less than 2% of the total cell population resulted positive for classical hematopoietic markers, such as CD34, CD45 or CD117. Surface expression of the SDF-1 receptor, CXCR4, instead, was established for 16% of the assessed cells. Nonetheless, as a whole, the available cells displayed a classical phenotypic signature of mesenchymal precursors; they were thus retained representative of the population of stromal stem progenitor cells, either residing within or able to

migrate to lesion sites, and involved in the regeneration of damaged mesenchymal tissues.

3.3.2 Effects of SDF-1 prestimulation on CXCR4 receptor expression

Real Time PCR analysis showed up-regulation of transcript for CXCR4 receptor after exposure of hMSC, in 3D matrices, to a higher SDF-1 factor concentration (figure 1 A).

Transcript data was paralleled by detailed cytofluorimetric analysis, that showed higher level of intracellular expression of thee receptor respect to the hMSC not affected by a prestimulation with 200ng/ml of SDF-1 (figure 1 B).



A B

Figure 1. Expression of CXCR-4 in BMSC after SDF-1 stimulation. Results show that exposure of stem cell to SDF-1 affects both mRNA production and the intracellular expression of CXCR-4 receptor.

3.3.3 Effect of SDF-1 stimulation on expression of motility relative genes

Since SDF-1 caused a chemtactic response of hMSC, our main aim was to identify molecules that were related to hMSCs movement. Real Time PCR

analysis highlighted that 3D culture systems strongly restore the expression of specific genes related to stem cells migration, but stimulation with SDF-1 do not affect levels of transcript (figure 2).

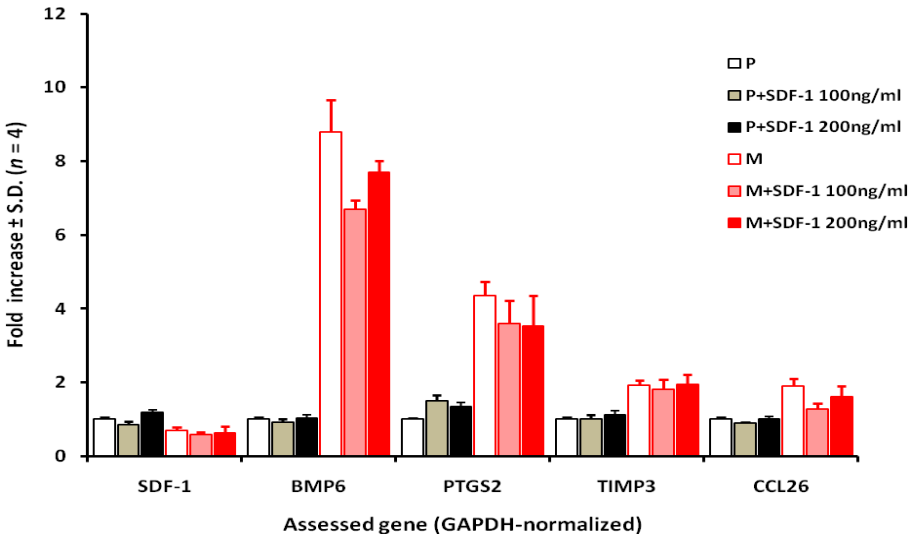
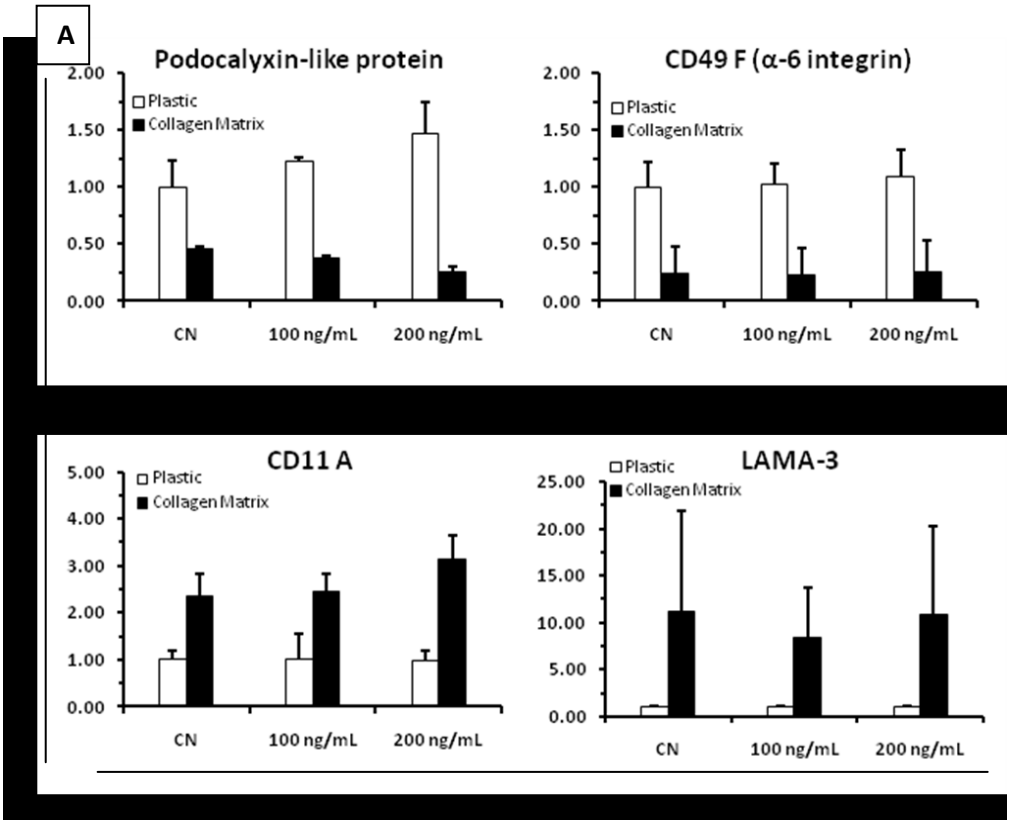


Figure 2 Real time analysis for genes with increased expression after 3D culture.

Real Time PCR analysis showed slight up-regulation of transcript for Podocalyxin-like protein and CD49F (α–6 integrin) genes, in hMSC seeded on plastic and stimulated with SDF-1 respect to the same cells cultured and stimulated into collagen matrix (figure 3 A).

Conversely when hMSC are seeded into collagen lattices the stimulation with SDF-1 was translated with up-regulation of CD-11A and LAMA-3 transcript (figure 3A). Stimulation with SF-1 (200ng/ml), of hMSC, seeded on plastic, strongly affect the mRNA production of several genes correlated with adhesion and motility phenomena (figure 3B)



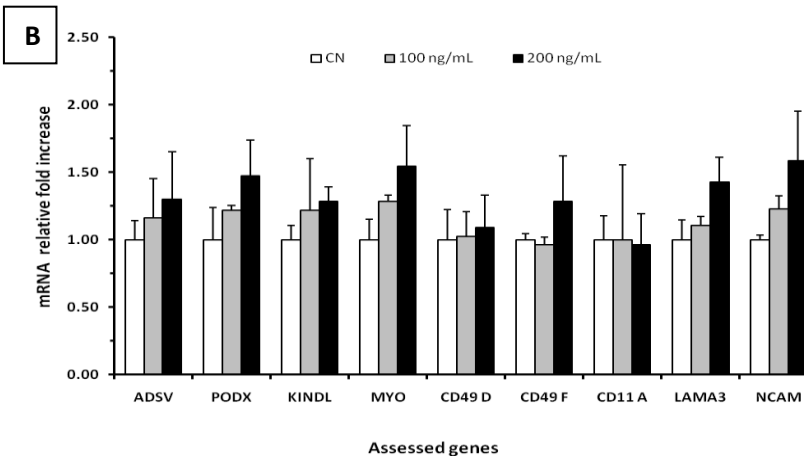


Figure 3 Effect of stimulation with SDF-1 on mRNA production of genes relative to adhesion and migration. Panel A: Relative increase of transcript for Podocalyxin-like protein; CD49-F; CD11; LAMA3 in hMSC cultured on plastic or in collagen matrix.
Panel B: Effect of SDF-1 stimulation on mRNA production for: Adservin (ADSV); Podocalyxin-like protein; Kindlerin (KINDL), Myosin-I-α (MYO); CD49-D; CD49F; CD11A; LAMA3; N-CAM-1

3.3.4 Effect of SDF-1 prestimulation on migratory parameters

According to the concentration of SDF-1 used, the experiment will be referred as “control” (without SDF-1 gradient), “100 h” (100ng/ml isotropic condition), “200 h” (200ng/ml isotropic condition), “100 no prest” (100ng/ml anisotropic

condition, without prestimulation with SDF-1), “200 no prest” (200ng/ml anisotropic condition, without prestimulation with SDF-1), “100+100” (100ng/ml anisotropic condition, with prestimulation with SDF-1 at 100ng/ml), “100+200” (200ng/ml anisotropic condition, with prestimulation with SDF-1 at 100ng/ml), “200+100” (100ng/ml anisotropic condition, with prestimulation with SDF-1 at 200ng/ml), “200+200” (200ng/ml anisotropic condition, with prestimulation with SDF-1 at 200ng/ml).

Windrose plots of the hMSC trajectories are reported in figure 4. In the absence of SDF-1, or in isotropic conditions cells move randomly (figure 4 A-B-C), whereas in presence of the SDF-1 gradient hMSC predominantly migrate toward the chemoattractant source (figure 4 D-E-F-G-H-I). Most of the cell tracks are co-aligned with the horizontal axis, i.e. the direction of SDF-1 gradient, particularly in the 100+100 conditions. Moreover, the SDF-1 gradient affects cells directional persistence. In fact, hMSC tracks are highly scattered in the control, whereas they are smoother when the gradient is present.

The effects of SDF-1 gradient on the migration speed values are indicated in figure 5 A and B for the values of S_{random} and S_{bias} respectively. Although the average value of S_{random} increases with the concentration gradient slope, no significant differences are observed among S_{random} values. On the other hand, a significant difference of S_{bias} is observed among the experimental setups.

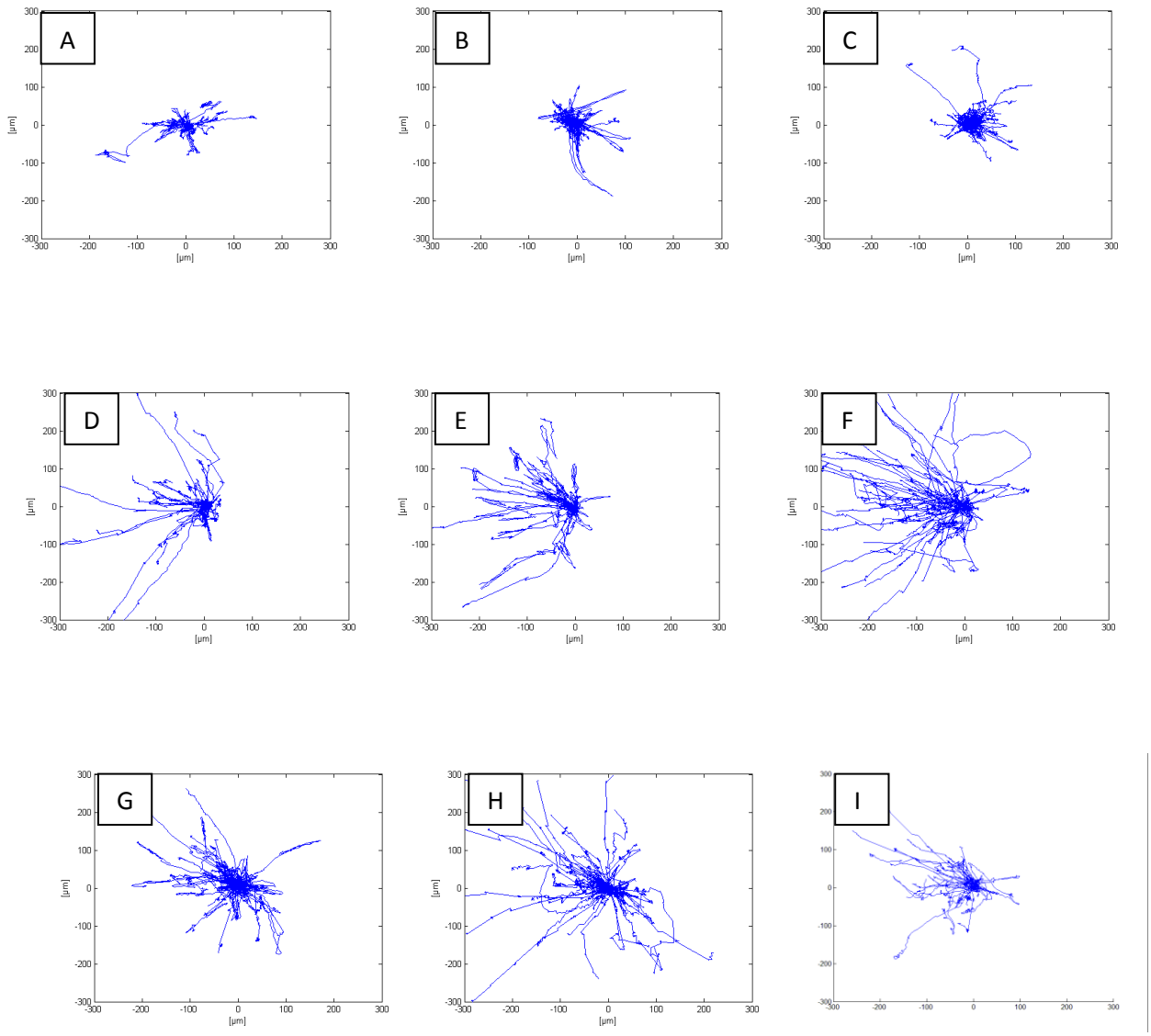
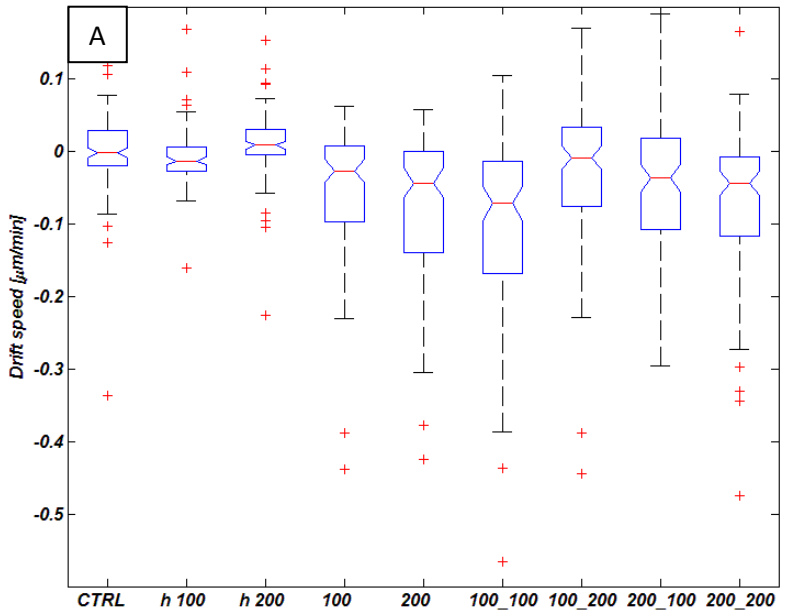


Figure 4 Windrose plots of hMSC trajectories recorded in the 24 h cell tracking experiment. A: control, B: 100h; C:200h, D: 100 no prest, E: 200 no prest, F:100+100, G:100+200, H: 200+100, I: 200+200.



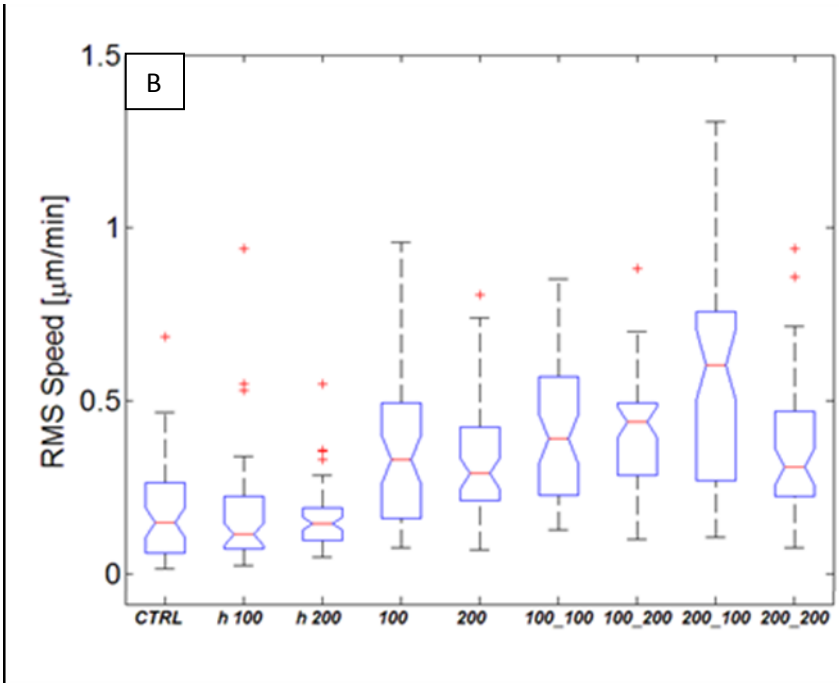


Figure 5 Box and whiskers plots of the migration parameters, evaluated for hMSC cells in the 9 different conditions.

Discussion

Crucial for the improvement of stem cells based therapies is to gain further insight into processes involving migration, homing and engraftment of MSC, all of which are fundamental for in situ tissue engineering strategies.

Our previous study highlighted the chemotactic effect of SDF-1 gradient on hMSC in three-dimensional collagen matrices.

Our results showed that the CXCR4 transcript is strongly expressed by hMSC, however, the surface expression of the CXCR4 antigen was quite low, confirming the findings of others (Kortesidis et al. 2005, Wynn et al 2004) and suggesting that this protein can be expressed intracellularly rather than on the surface. The majority of CXCR4 (98%) is localized in endosomal compartments and cycles continuously to and from the cell surface via endocytosis involving clathrin-coated pits (Zhang et al. 2004), and we can assume that CXCR4 sequestered intracellularly in MSCs is mobilized to the cell surface, during cytokine stimulation.

Here we showed that in addition to its role in mediating cell migration, SDF-1 may also play important autocrine roles. We demonstrated that hMSC are able to synthesis this chemokine, which may act in an autocrine manner via CXCR-4 (figure 1). The up-regulation of CXCR-4, after SDF-1 stimulation is reflected in a different migratory behavior of hMSC in response to a SDF-1 gradient.

Interestingly the exposure of hMSC, seeded in collagen lattices, to SDF-1 induces the down-regulation of CD11A and LAMA3, respect to the same cells cultured on plastic, in which we can observe an up-regulation of the transcript for the same genes . This observation seems to recapitulate the in

vivo phenomena in which cytokines that are up-regulated under injured conditions are released into circulation from remote tissues, stimulating MSC to down-regulate the adhesion molecules that hold them at their niche. Phenomenon that is impossible to realize when the cell adhere to the plastic, a situation too far away from the in vivo conditions (figure 3 A).

The stimulation with SDF-1 in 3D environment appears to disadvantages the up-regulation of same genes involved in the proliferation activity, that is, instead, advantage in culture conditions on plastic (figure 3A). Moreover when cell are cultured on plastic the exposure to SDF-1 (200ng/ml) give rise to up-regulation of Adservin, Podocalyxin-like protein, Kindlerin, Myosin-I-α, CD49-D, CD49F, CD11A, LAMA3, N-CAM-1, genes involved in adhesion and migration of stem cells (figure 3B).

Curiously the short-term (24 h) culture in tridimensional collagen matrix resulted in up-regulation of several genes involved in cell migration (figure 2).

Bone morphogenetic protein-6; known to regulate migration of vascular smooth muscle cells and of immature neurons.

Prostaglandin endoperoxide synthase -type 2; inducible isoenzyme, known to be up-regulated after CXCL8 –, or progesterone-, or BMP6 administration.

TIMP3: Tissue inhibitor of Matrix metalloprotease 3;in vitro culture confluence reduces migration:reactivation of proliferation/motility depends upon matrix degradation, thus upon impairement of TIMP3 expression. Uprisal in TIMP3 mRNA correlates with differentiation.

CCL26 (eotaxin-3):Known to regulate chemotaxis of basophil and eosinophils.

The microenvironment has to be regarded as an informative milieu in which cultured cells should find proper clues to drive their functions and/or

differentiation pathways, ultimately improving their outcomes in cell-based tissue regeneration attempts.

Our results underlined two different roles of SDF-1. This chemokines is able to strongly chemoattract hMSCs in tridimensional collagen matrix. The event is particularly enhanced for MSC, affecting different aspects of cellular motility namely the shape of the paths, the bias component and the random component of the speed. However, SDF-1 displays autocrine effects via CXCR-4; indeed the stimulation of hMSC with SDF-1 results in the up-regulation both of CXCR-4 receptor transcript production and intracellular expression. This evidence is translate in a alteration of migratory parameters of MSC in response to SDF-1 gradient.

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CHAPETER 4

Peptide activated materials to recruit circulating stem cells

4.1 Introduction

In order to achieve successful regeneration of damaged tissue or organs, the development of biomaterials for TE applications has recently focused on the design of biomimetic materials that are able to interact with the cell by biomolecular recognition.

The design of biomimetic materials is an attempt to make the materials such that they are capable of eliciting specific cellular responses mediated by specific interactions, which can be manipulated by altering design parameters. Biomolecular recognition of materials by cells can be achieved by several design strategy. One of these involves incorporation of cell-binding peptides on biomaterials surface. The immobilization of signaling peptides gives, for example, the surface of biomaterials adhesive for a specific cell population. Indeed the ability to identify and isolate specific stem cells from heterogeneous populations is of fundamental importance in order to develop novel stetegy for TE and regenerative medicine applications. As matter of fact in order to effectively allow clinical implemetation of several experimental procedure is necessary to target specific cells that are effectively able to regenerate tissue/organ defects.

In particular, the development of single-cell tools would allow the detection of MSC cells in blood for in situ Tissue Engineering applications. Moreover, a deeper understanding of what signals can trigger specific actions will be necessary in order to create novel stem cells based therapies, which rely upon the selection and characterization of stem cell populations identified in numerous adult tissues.

The first step in creating a biomimetic surface, useful for TE applications, is the development of suitable surface coatings allowing efficient coupling of biologically active molecules on the device. Such coatings can be obtained through self-assembly of alkanethiol monolayers (SAMs) (Ostuni et al. 1999, Zhang et al. 2003, Palyvoda et al. 2008).

Self-assembled monolayers (SAMs) of alkanethiols on gold are common model systems for biological and biotechnological applications because of their unique set of attributes (Yamauchi et al. 2004; Wegner et al. 2004). SAMs can be designed to be "bioinert", and therefore resistant to nonspecific protein adsorption. They can also be terminated with a wide variety of functional groups, which enables variation in their surface reactivity, charge density, hydrophilicity (Harder et al. 1998; Li et al. 2005; Boozer et al. 2006). Finally, the molecular structure of SAMs results in a well-defined density of functional groups on the surface (Houseman et al 2003; Shwartz et al. 2001; Lee et al. 2007), without compromising their structure or activity (Du et al. 2005). This is particularly important in mechanistic studies of intermolecular interactions, such as receptor-ligand interaction. On the basis of these attributes, alkanethiol monolayers formed on gold-coated glass substrates have been used as model systems for exploring several biological phenomena, including cell adhesion (Houseman et al. 2004; Roberts et al. 1998) cell migration (Liu et al. 2007), and the analysis of intermolecular interactions (eg., protein-protein and DNA-DNA interactions) (Wegner et al. 2005; Hokahata et al. 1998, Gong et al. 2006).

SAMs have been formed on a variety of surfaces, including glass (Banga et al. 2005), silicon (Wasserman 1989), gold (Pale-Grosdemange et al. 1991),

diamond (Lasseter et al. 2004), and amorphous carbon (Sun 2006). SAMs formed via coordination of alkanethiolates onto gold-coated substrates are of particular interest in biochemical applications, as they are formed easily and rapidly (Bain et al. 1989), are stable in polar solvents (Flynn et al.2003).

The latest approaches in tissue-engineered repair of bone suggest to potentiate the self-healing properties displayed by tissues and progenitor cells within the host. In this light, materials able to selectively recruit mesenchymal stem cells, the ones deputed to bone repair could potentiate the self-healing capacities of a patient directly within the lesion sites. Our research aims at creating an innovative device that will isolate Mesenchymal Stem Cells from different sources.

Literature studies shown that synthetic peptide derived from the second cysteine rich domain (184-204 aa) of the canonical Wnt inhibitor Dickkopf-1 (DKK-1) has utility in controlling the growth and recovery of hMSCs from bone marrow stroma. DKK-1 binds LPR6 receptor via its carboxyl-terminal cystein rich domain.

Monocyte chemotactic Protein-1 (MCP-1) is a member of CC chemokine family. Peptide (11-38 aa) derived from its aminoacidic sequence, binds CCR2 receptor. It has been hypothesized that MCP-1 levels increase after ischemic brain providing signals required to promote adhesion and migration of monocytes and lymphocytes, but also promotes migration of MSCs into the lesion.

In this study we present a call for hightroughput signals presentation assay that allow the possibility of rapid, specific and detailed analysis of cell population.

4.2 Materials and methods

4.2.1 Cell culture

Human mesenchymal stem cells (hMSC) were purchased from Lonza Inc. (Walkersville, MD, USA). Three hMSC aliquots were pooled to reduce bias from samples derived from single donors, rinsed twice with PBS and plated at a final density of 2-5x10^6 cells/100 mm dish in Coon's modified Ham's F12 medium (Biochrom AG, Berlin, Germany), supplemented with 10% FBS and 1 ng/ml FGF-2 (LiStarFish, Milan, Italy) (1, 2). Cells were maintained in culture and used within their 3rd passaging (P1-P3). All cultures were kept in a humidified atmosphere at 37°C and 5% of CO_2; the medium was replaced every 3 days. Whenever needed cells were trypsinized by standard protocols, washed in PBS and resuspended to the desired concentration for further use.

4.2.2 Aminoacidic sequences of synthetic peptides

MCP-1: CCYNFTNRKISVQRLASYRRITSSKCPK

DKK-1: LSSKMYHTKGQEGSVCLRSS

4.2.3 Peptides binding assay

To assess the affinity of the synthetic peptides (MCP-1 and DKK-1), hMSCs were cultured onto chamber slides and incubated at 37 °C and 5%CO_2. After 24h of incubation cell were fixed with 4% paraformaldeyde, for 15 min at RT, rinsed twice with PBS buffer and incubated with PBS-BSA 0.5% to block unspecific binding. Monolayers were then incubated with peptides FITC conjugated (40 μg/ml), prepared in a solution of PBS-BSA 0.5% for 1h.

Finally, samples were rinsed 3 times with PBS and observed by confocal microscopy, using a 10X objective.

Affinity analyses were performed by means of a confocal microscope Zeiss LSM 510, equipped with an argon laser, at a wavelength of 488 nm and a He–Ne laser at a wavelength of 543 nm and objectives 10X. Images were acquired with a resolution of 512X512 or 1024X1024 pixel. The emitted fluorescence was detected using filters LP 505, BP 560–600 and HFT 488/543 for FITC.

4.2.4 Preparation of a Monolayer on Gold Substrate

Gold substrates (purchased from Arrandee Company) were sonicated in ethanol for three minutes and washed with isopropanol to remove all organic contaminants. The gold substrates were dried under a stream of nitrogen prior to monolayer formation. Monolayers were formed by incubating cleaned gold substrates in inert atmosphere in an N-methylpyrrolidone anhydrous solution containing mixture of NHS ester monothiolalkane(C11)PEG6-NHS ($(EG)_6NHS$) and (11-mercaptoundecyl) triethyleneglycol, $(EG)_3OH$ at various molar rations (2 mM total thiol concentration) overnight (figure 1). In particular were prepared mixed alkanethiol solutions containing:

1. 0 % $(EG)_3$ OH and 100 % $(EG)_6NHS$
2. 100 % $(EG)_3$ OH and 0 % $(EG)_6NHS$
3. 99% $(EG)_3$ OH and 1 % $(EG)_6NHS$
4. 98% $(EG)_3$ OH and 2 % $(EG)_6NHS$
5. 95% $(EG)_3$ OH and 5% $(EG)_6NHS$

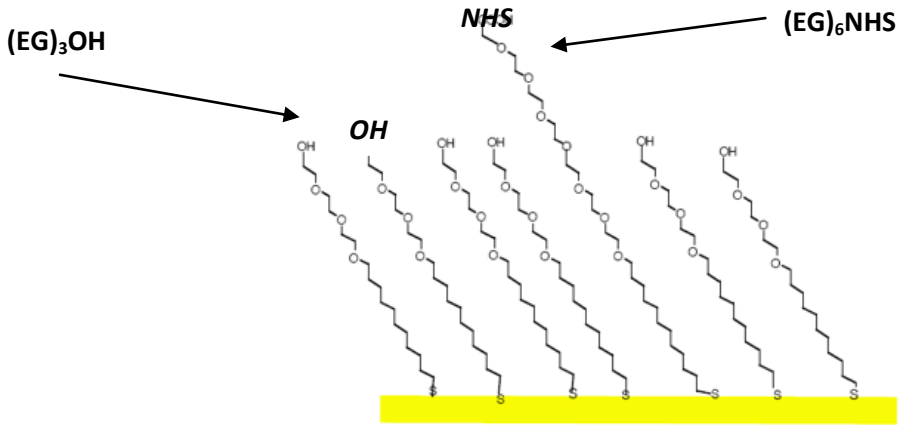

Figure 1. Mixed SAMs using solution that contain both: $(EG)_6NHS$ and $(EG)_3OH$ terminated alkanethiolates

Next day the slides were washed once with isopropanol anhydrous, once quickly with ethanol (to condition the slides), then rinsed again with isopropanol and they were blow dried with argon. After SAM deposition on SAM 95% $(EG)_3$ OH and 5% $(EG)_6NHS$, each peptide was immediately covalently attached onto SAM surfaces incubating the activated SAM with 1 mg/mL of peptides in PBS solution (Figure 2).

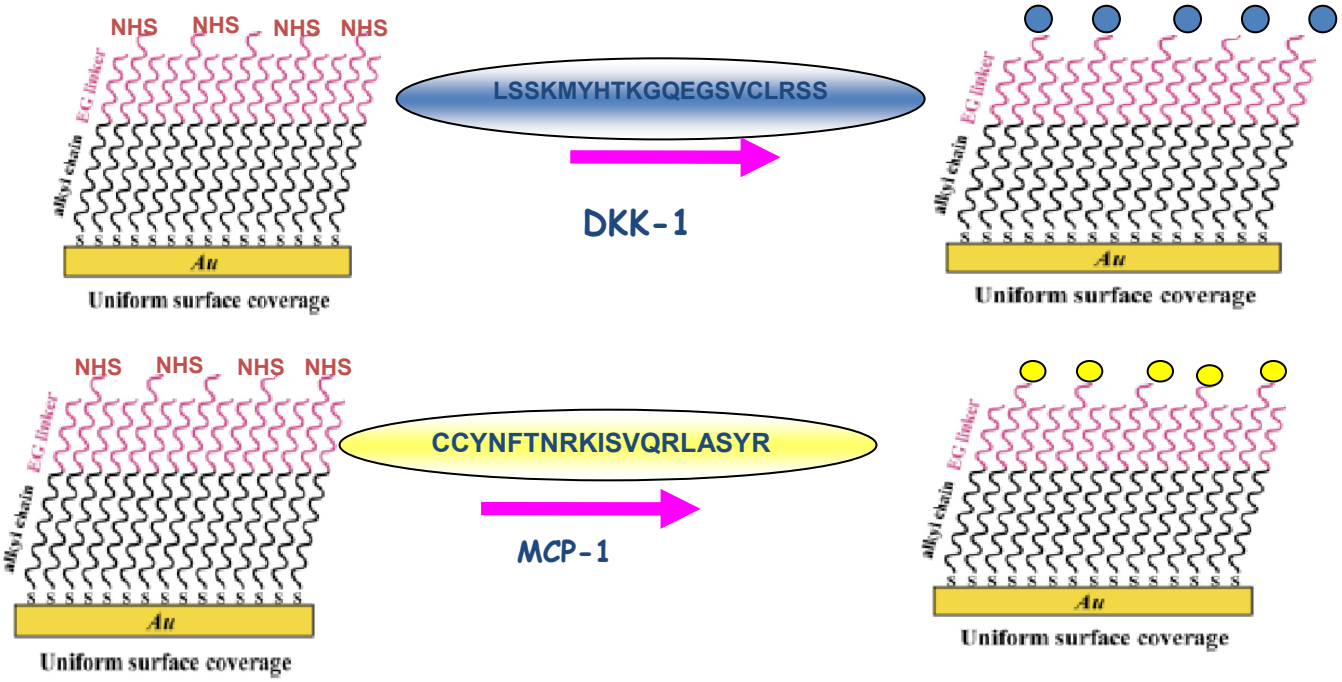

Figure 2 Chemical immobilization of peptides on mixed SAMs

Furthermore to avoid the nonspecific binding of cellular receptors to the non reacted NHS function, the SAM surface monolayer was treated with hydroxylamine solution ($NH_2CH_2CH_2OH$). The negative control used were:

1. SAM 100 % $(EG)_3$ OH and 0 % % $(EG)_6$NHS
2. SAM 0 % $(EG)_3$ OH and 100 % % $(EG)_6$NHS treated with $NH_2CH_2CH_2OH$ (excess)
3. 95% $(EG)_3$ OH and 5% % $(EG)_6$NHS treated with $NH_2CH_2CH_2OH$ (excess)

XPS studies on the SAMs

1. 0 % $(EG)_3$ OH and 100 % $(EG)_6$NHS
2. 100 % $(EG)_3$ OH and 0 % $(EG)_6$NHS
3. 99% $(EG)_3$ OH and 1 % $(EG)_6$NHS
4. 98% $(EG)_3$ OH and 2 % $(EG)_6$NHS
5. 95% $(EG)_3$ OH and 5% $(EG)_6$NHS

Detected composition of the carbon, oxygen and sulfur on the surface close to the theoretical values of these constituents in the SAM (data not shown). Moreover the surface properties were verified and the values obtained are in agreement with those reported in literature (Bain, C. D.; Whitesides, G. M. Langmuir 1989, 5, 1370-1378). Variable-angle XPS was used to obtain information on SAM composition and assembled alkanethiol orientation. Values obtained are in agreement with theoretical calculations, within the sensitivity of the instrument. Comparing atomic percentages at multiple depths of analysis indicated that SAMs:

1. 0 % $(EG)_3$ OH and 100 % $(EG)_6$NHS

2. 100 % $(EG)_3$ OH and 0 % $(EG)_6$NHS
3. 98% $(EG)_3$ OH and 2 % $(EG)_6$NHS
4. 95% $(EG)_3$ OH and 5% $(EG)_6$NHS

were oriented correctly, with the sulphur atom at the greatest depth from the surface an the functional tail groups being closest to the surface.

4.2.5 Cell sorting assay

In order to demonstrate that DKK-1 and MCP-1 synthetic peptides, are able to recovery hMSCs, $2x10^6$ cells were injected in a purposely designed close-system bioreactor (Figure3), providing chambers to hold the functionalized slides. A steady flux (80rpm) of serum-free culture medium through all chambers was maintained for 45 minutes by the use of a multichannel Watson-Marlow 323 peristaltic pump. The functionalized slides were then washed in PBS and cells were fixed in 4% PFA.

Standard immunofluorescence staining, using monoclonal anti-human CD44 (Acris Antibodies, Herford, Germany), anti-human Actin, anti CD-146 (BD Biosciences, San jose, CA, USA) were revealed respectively by goat-anti mouse PE- or FITC-conjugated IgG (Jackson Immunoresearch Lab. Inc., West Groove, PA, USA); DAPI was used as nuclear staining.

Blockade of unspecific sites was performed incubating slides with goat serum diluted in PBS (1:10). Five images (20x enlargements) for each surface were acquired on a BX51 Olympus microscope; cell number and

nucleus/cytoplasm area ratios were calculated analyzing acquired images with ImageJ free software (version 1.38; http://rsb.info.nih.gov/ij/).

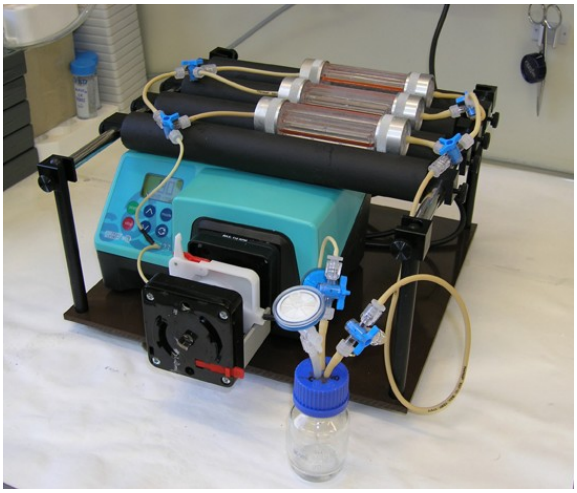

Figure 3. Close -system bioreactor

4.3 Results

4.3.1 Peptide binding assay

In order to evaluate the affinity of hMSCs by MCP-1 and DKK-1 synthetic peptides, we incubated cell monolayers with peptides. On inspection of the stained monolayers with confocal microscope, binding of hMSCs by MCP-1 and DKK-1 was evident (Figure 4 A-B; C-D). The control was carried out in the absence of peptides and no fluorescence is detectable (Figure 4 E-F).

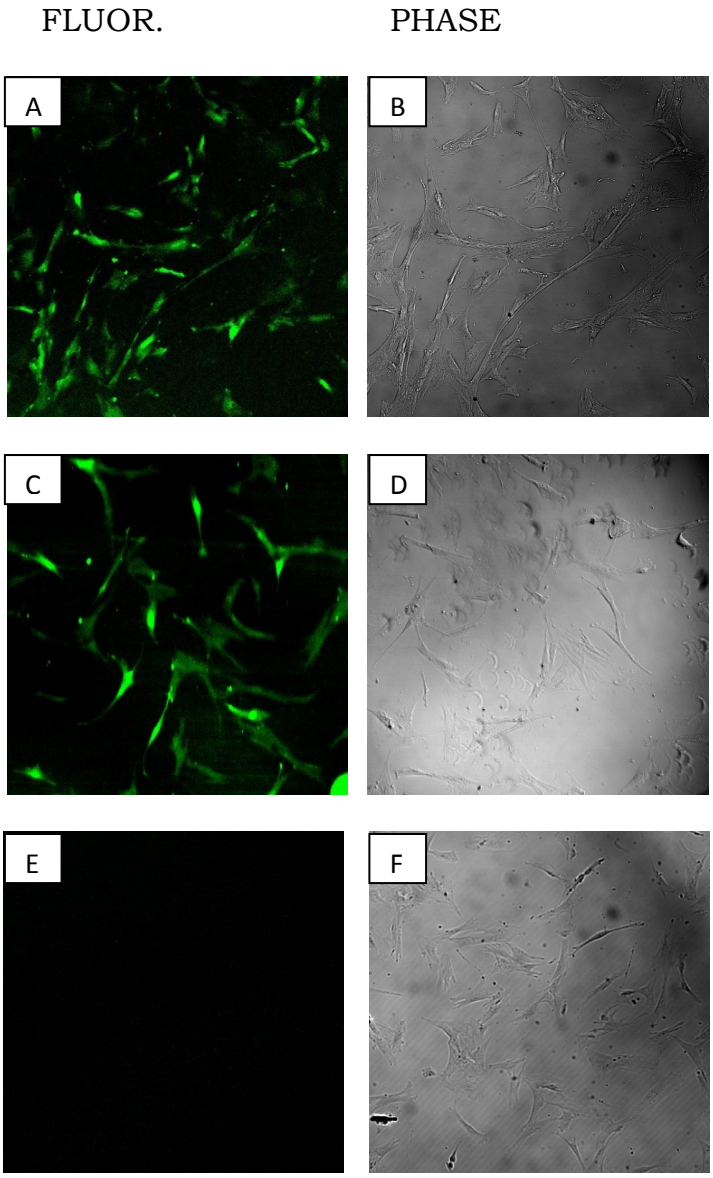

Figure 4. Representation of peptide binding assay. Fluorescent signal is presented on the left and phase images on the right. The binding was visualized by fluorescence, for synthetic peptide derived from MCP-1 (A-B) and DKK-1 (C-D). The control was carried out in the absence of peptides (E-F).

4.3.2 Cell sorting assay

In order to evaluate the cell recovery properties, each functionalized surface were exposed to a constant flux of human bone marrow stromal cells in a bioreactor, for 45 minutes.

We observed that peptides immobilization is required to obtain cell attachment on the SAM-functionalized gold substrates. In the absence of MCP-1 or DKK-1 peptides cell failed to attach on surfaces. To establish the efficiency of trapping on functionalized surfaces, we stained and counted the nuclei on each acquired images. Interestingly the largest amount was found onto MCP-1-treated materials (mean±S.D. per visual field: 176±10; n = 5; p<0,001), (Figure 5).

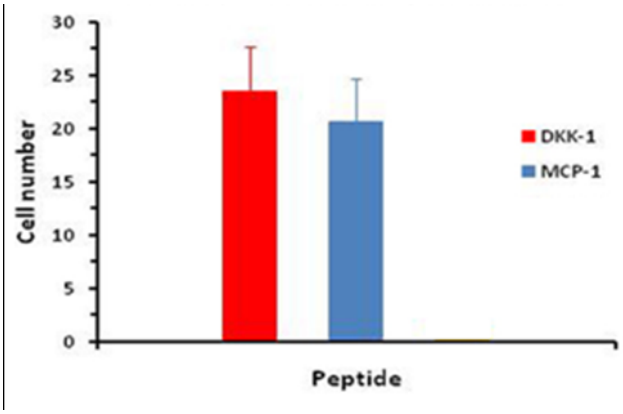

Figure 5. Average cell number per visual field of MSC attached on surface functionalized with DKK-1 or MCP-1

The morphology of the attached cells, for MCP-1- treated surfaces varied from that of the cells attached onto DKK-1 functionalised materials, as demonstrated by the different ratio of the nucleus/cytoplasm area. The cells attached on surface functionalized with MCP-1 synthetic peptide showed a smaller ratio between area nucleus/citoplasm, demonstrated a bigger

dimension of these cell respect to the cells attached on surfaces functionalized with DKK-1 peptide (Figure 6). Different dimensions are evident also in fiugure 7 (A-B).

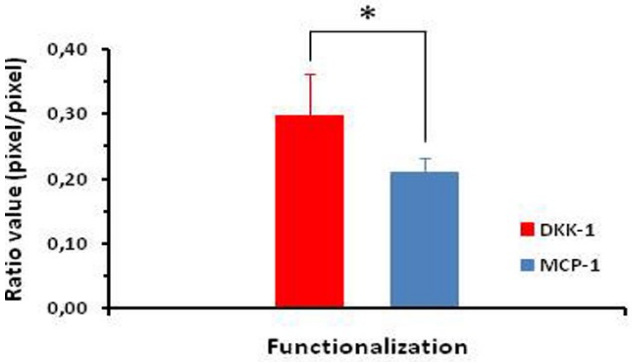

Figure 6. Nucleus/cytoplasm surface ratio of MSC on treated surfaces with MCP-1 or DKK-1, after 45 min under flux *p<0,05; n>100

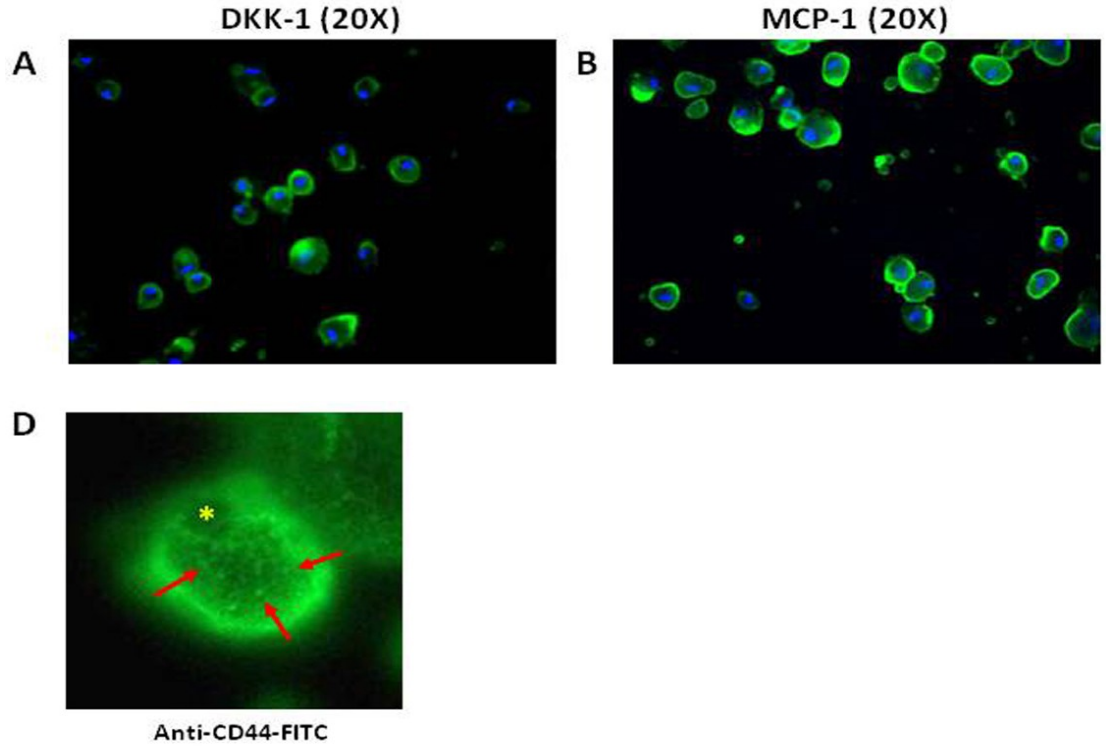

Figure 7. Merged fluorescence images (anti-CD44 in green and nucleus in blu) of hMSCs on surfaces functionalized with DKK-1 (A) or MCP-1 (B). In D the red arrows evidence the presence of clusters of CD44 receptor on hMSCs surfaces, the asterisk indicate the position of nucleus.

Interestingly immunofluorescence assay showed that cell adherent to both MCP-1 and DKK-1 functionalized surfaces are positive for the expression of CD146, a relevant marker for the identification of osteogenic precursors able to regenerate also a complete and functional marrow (Sacchetti *et al.* 2007) (figure 8 and 9).

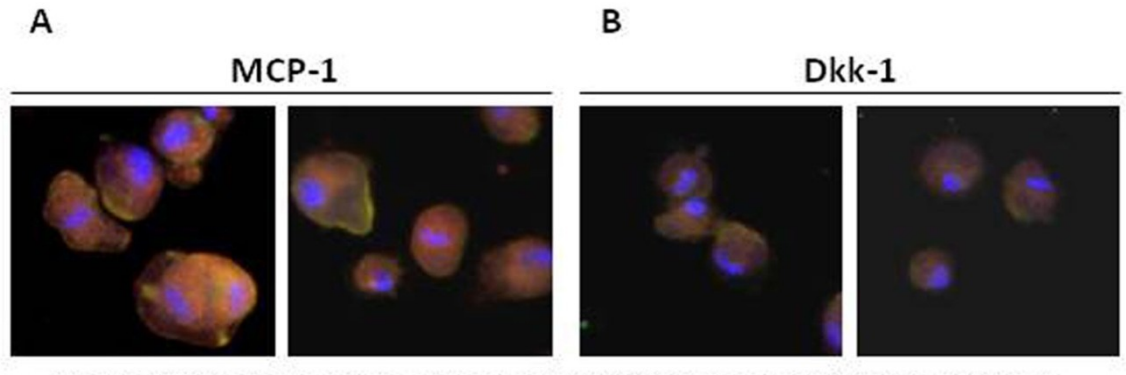

Figure 8. Merged fluorescence images (anti CD-44 FITC and antiCD-146-PE) of hMSC adherent to functionalized slides with MCP-1 (panel A) or with DKK-1 (panel B)

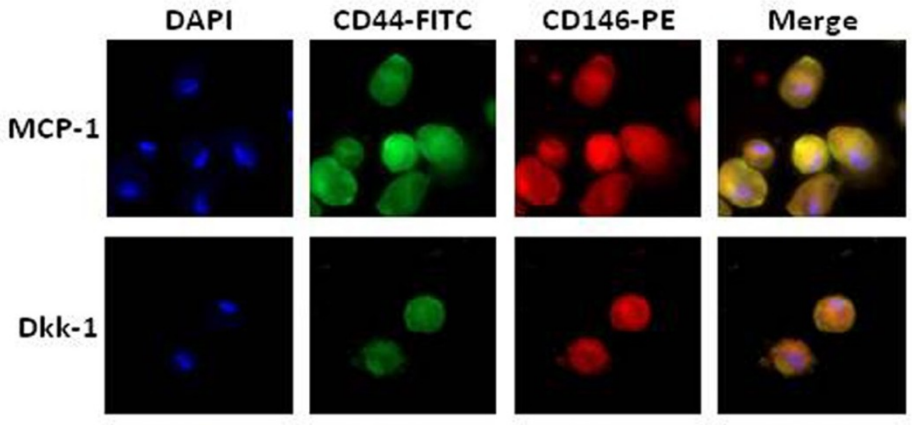

Figure 9 Immunofluorescence images of hMSC adherent to functionalized slides with MCP-1 or with DKK-1.

The staining of cytoskeleton of hMSCs linked on functionalized surfaces with MCP-1 or DKK-1 peptides showed that the area of cells is effectively crossed by a network of actin fibers (green arrows) that unfolds over the edge of

overlapping colors anti-actina/anti-CD44 (areas and arrows Merged images in orange) (figure 10).

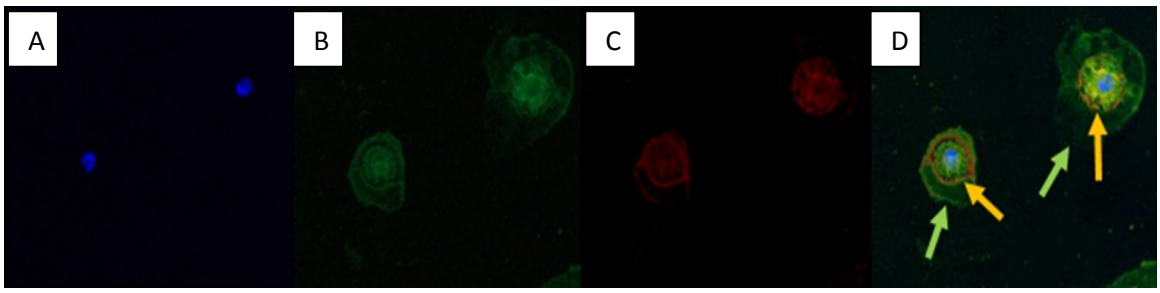

Figure 10. Staining of hMSC adherent to functionalized slides with MCP-1. Nucleus in blu (A), cytoskeleton in green (B), anti-CD44 in red. In D there is a merged of three stainig, actin fibers (green arrows) unfolds over the edge of overlapping colors anti-actina/anti-CD44 (areas and arrows Merged images in orange).

4.4 Discussion

The use of sequences derived from DKK-1 and MCP-1 seems to drive the selection of specific cell types, proving the possibility to trap progenitor cells of mesenchymal tissues onto the treated surfaces. Thus these surface modifications may be foreseen as possible implementation of the materials used for prosthetic-based approaches in bone repair, provided that studies under way allow the determination of the phenotype and the differentiation potential of the trapped cells.

In particular, the number of cells sequestered by the surfaces functionalized with MCP-1 synthetic peptides is higher than number of cells attached on DKK-1 functionalized slides.

Furthermore cells spread on the MCP-1 activated surfaces in a more effective way respect to the cells that interact with DKK-1probably due to a better adhesion of cells.

Interestingly immunofluorescence assay showed that cell adherent to both MCP-1 and DKK-1 functionalized surfaces are positive for the expression of CD146, a relevant marker for the identification of osteogenic precursors able to regenerate also a complete and functional marrow (Sacchetti *et al.* 2007) (figure 8 and 9). This is of fundamental importance to improve the selection of a specific cells population that are able to effectively differentiate and regenerate tissue damage.

Our results represent a first step for the realization a more complex device able to select and trap Mesenchymal Stem from heterogeneous population. For therapy development, this kind of biomimetic scaffold could represents a fast and efficient clinical platform technology for purifying and further characterising heterogeneous populations. The ability to target cell-surface markers expressed selectively by distinct cell types is particularly useful for the enrichment of stem cells. The isolation of “rare cells” is, of course, entirely dependent on their identification.

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